GLUCANSUCRASES OF LACTOBACILLI:

Characterization of genes, enzymes, and products synthesized

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Characterization of genes, enzymes, and products synthesized

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Co-promotor:	Dr. G.H. van Geel-Schutten
Beoordelingscommissie:	Prof. Dr. B.W. Dijkstra
	Prof. Dr. D.B. Janssen
	Prof. Dr. J. Kok

Voorwoord

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SLAVKO

Contents

Chapter 1

General introduction: Glucansucrases of lactic acid bacteria

S.A.F.T. van Hijum, S. Kralj, L.K. Ozimek, L. Dijkhuizen and G.H. van Geel-Schutten

Part of this chapter has been submitted for publication

INTRODUCTION

Carbohydrates, $C_n(H_2O)_n$ constitute one of the four major classes of biomolecules, together with proteins, nucleic acids, and lipids and comprise most of the organic matter on earth (Stryer, 1995). The simplest carbohydrates are monosaccharides (aldehydes or ketones that have two or more hydroxyl groups). They can be linked through glycosidic bonds to form oligosaccharides (e.g. sucrose, maltotriose, panose), and polysaccharides (e.g. starch, dextran). Sucrose [α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] is a natural disaccharide, mainly found in sugar cane and sugar beet but also in many other plants, especially fruits. It lacks a free reducing group in contrast with most other sugars. Transglycosylation of sucrose (consisting of a glucose and fructose moiety) to α-glucan polymers by glucosyltransferase (glucansucrase) enzymes from lactobacilli is reviewed in this chapter.

EXOPOLYSACCHARIDES

Some lactic acid bacteria (LAB), including species of *Lactobacillus* are known to produce extracellular polysaccharides (EPS). Depending on their composition and mechanism of biosynthesis, EPS can be divided in two classes: homopolysaccharides (polymers composed of glucose or fructose units, respectively) or heteropolysaccharides. The latter are composed of a variety of sugar residues, mainly glucose, galactose, fructose and rhamnose. In some cases charged groups such as acetate, phosphate or glycerolphosphate are present (de Vuyst & Degeest, 1999).

Heteropolysaccharides of lactic acid bacteria

Intracellular sugar nucleotides, formed from intermediates of central carbon metabolism play an essential role in heteropolysaccharide synthesis. The activated sugar nucleotides are transferred onto a lipophilic carrier, followed by assembly of the oligosaccharide repeating unit by specific glycosyltransferases. Then the repeating unit is exported, polymerized and released (in the case of secreted polysaccharides) (Jolly *et al.*, 2002, Welman & Maddox, 2003).

Genes important for heteropolysaccharide synthesis are located either on plasmids or on the chromosome. In both cases EPS biosynthesis genes are organized in gene clusters which show a clear conservation in organization and sequence. The clusters carry genes involved in regulation, polymerisation/chain length determination, biosynthesis of the repeating unit (glycosyltransferases), polymerization and export (de Vuyst & Degeest, 1999, Jolly *et al.*, 2002, Welman & Maddox, 2003).

Homopolysaccharides of lactic acid bacteria

Homopolysaccharides of lactic acid bacteria (LAB) can be divided into two groups: **i**) β-fructans: **a**) Levans composed of mainly β-(2→6) linked fructose residues, are

synthesized by streptococci (Carlsson, 1970, Hancock *et al.*, 1976, Simms *et al.*, 1990), *Leuconostoc mesenteroides* (Robyt & Walseth, 1979) and two different lactobacilli: *Lactobacillus reuteri* 121 (van Geel-Schutten *et al.*, 1999, van Hijum *et al.*, 2001) and *Lactobacillus sanfranciscensis* LTH2590 (Korakli *et al.*, 2003). The lactobacilli *Lactobacillus frumenti, Lactobacillus pontis, Lactobacillus panis and Weissela confusa* also produce fructans, but the exact binding types have not been determined (Tieking *et al.*, 2003). Levan formation has also been observed for the non-LAB *Gluconobacter diazotrophicus* (Hernandez *et al.*, 1995), *Zymomonas mobilis* (Kyono *et al.*, 1995, Song *et al.*, 1993) and *Bacillus* spp. (Li *et al.*, 1997, Perez Oseguera *et al.*, 1996, Tanaka *et al.*, 1978). **b**) inulin containing β-(2→1) linked fructose molecules, are synthesized by (cariogenic) *Streptococcus* species, *Leunostoc citreum* CW28 and by an inulosucrase of *Lb. reuteri* 121 (Ebisu *et al.*, 1975, Shiroza & Kuramitsu, 1988, Rosell & Birkhed, 1974, van Hijum *et al.*, 2002, Olivares-Illana *et al.*, 2003).

ii) α-D-glucans, comprising the following five groups (Fig. 1): (**a**) reuteran, containing large amounts of α-(1→4) glucosidic bonds synthesized by *Lb. reuteri* 121 and *Lactobacillus reuteri* BioGaia (Kralj *et al.*, 2002, Kralj *et al.*, 2004a), (**b**) dextran, containing predominantly α-(1→6) linked glucopyranosyl units in the main chain (Cerning, 1990), (**c**) mutan, a polyglucose with mainly α-(1→3) linkages (various streptococci) (Hamada & Slade, 1980) and *Lactobacillus reuteri* ML1 (Kralj *et al.*, 2004b), (**d**) alternan with alternating α-(1→6) and α-(1→3) linked D-glucopyranosyl units (*Leuconostoc mesenteroides* NRRL B-1355) (Arguello-Morales *et al.*, 2000), and (**e**) glucan polymers containing large amounts of α-(1→2) linkages (predominantly α-(1→2,6) branching points), produced by *Leuconostoc mesenteroides* strain NRRL-B1299 and a mutant strain (R510) of *Ln. mesenteroides* NRRL B-1355 (Bozonnet *et al.*, 2002, Smith *et al.*, 1998). Within these five distinct groups the glucans may further differ in the nature and amount of other glucosidic linkages present, e.g. of the α-(1→2), α-(1→3), α-(1→4) or α-(1→6) type, the degree of branching, the type of branching point, e.g. with α-(1→2,6) to α-(1→3,6), or α-(1→4,6) glucosidic linkages, molecular weight, the length of the branching chains and their spatial arrangement (Monchois *et al.*, 1999d). As a result, there are large variations in solubility and other physical characteristics of the glucans (Monchois *et al.*, 1999d).

Other homopolysaccharides synthesized by LAB are β-glucans produced by *Pediococcus spp.* and *Lactobacillus* spp. (Llauberes *et al.*, 1990, Duenas-Chasco *et al.*, 1998) and galactan synthesized by *Lactococcus lactis* subspecies cremoris H414 (Gruter *et al.*, 1992). However these polysaccharides are synthesized in the same way as heteropolysaccharides and sucrase enzymes are not involved in their synthesis.

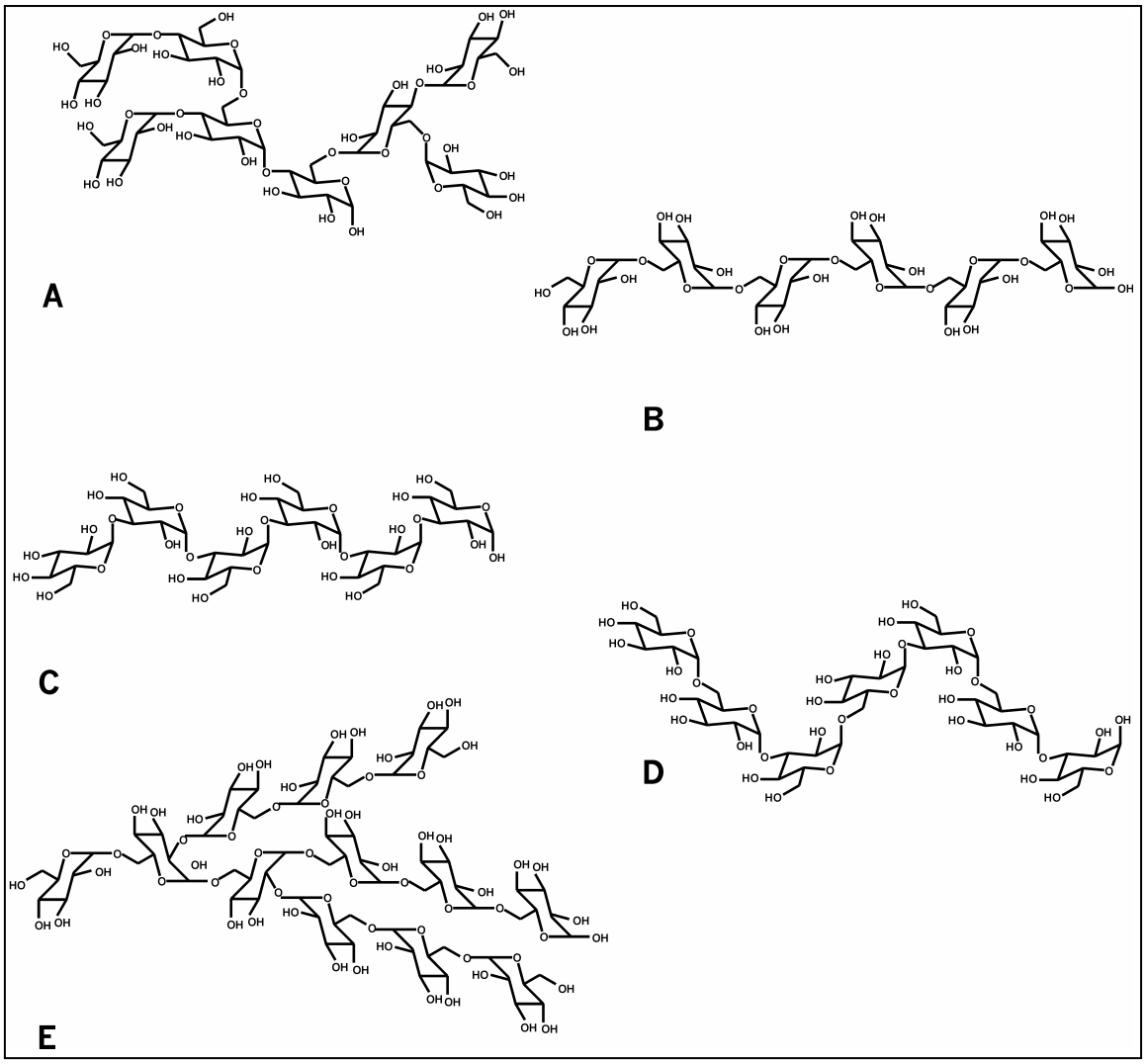


Figure 1. Schematic structures of various LAB α-glucans: reuteran α-(1→4)/α-(1→6) (**A**), dextran α-(1→6) (**B**), mutan α-(1→3) (**C**), alternan α-(1→3) / α-(1→6) (**D**), and dextran containing α-(1→2) branch linkages (**E**).

FRUCTAN SYNTHESIZING ENZYMES

Bacterial fructans are synthesized by sucrase type of enzymes, the fructosyltransferases (FTFs). These extracellular enzymes cleave their substrate sucrose (or in some cases raffinose) and use the energy released (between the glucose and fructose) to couple a fructosyl unit to a growing fructan (polyfructose) chain, to sucrose, water (hydrolysis), or other acceptors. Inulin is synthesized by inulosucrase (sucrose: 2,1-β-D-fructan 1-β-D fructosyltransferase; E.C. 2.4.1.9) and levan is synthesized by levansucrase (sucrose: 2,6-β-D-fructan 6-β-D fructosyltransferase; E.C. 2.4.1.10).

GLUCAN SYNTHESIZING ENZYMES

Glucansucrases, (commonly named glucosyltransferases; GTFs); are large extracellular enzymes capable of synthesizing various glucans (dextran, mutan, alternan and reuteran; Fig. 1) from sucrose. For example, dextran is synthesized by dextransucrase (sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferase; E.C. 2.4.1.5) and alternan is synthesized by alternansucrase (sucrose:1,6(1,3)-α-D-glucan 6(3)-α-D-glucosyltransferase; E.C. 2.4.1.140). Recently, an inulosucrase with structural features of both glucosyltransferases and fructosyltransferases was isolated from *Ln. citreum* CW28 (Olivares-Illana *et al.*, 2003).

Two different reactions are catalyzed by glucansucrase enzymes, depending on the nature of the acceptor: i) hydrolysis, when water is used as acceptor; ii) glucosyl transfer (transferase), which can be divided in: a) polymerization, when the growing glucan chain is used as acceptor, and b) oligosaccharide synthesis, when oligosaccharides (e.g. maltose, isomaltose) are used as acceptor. The bond between the glucose and fructose moiety from sucrose provides the energy for catalysis and formation of a new glycosidic bond.

α-Glucan synthesis has been observed in four different genera of lactic acid bacteria: streptococci, leuconostocs, weissella and lactobacilli (Monchois *et al.*, 1999d, Kralj *et al.*, 2004b, Kralj *et al.*, 2004a, Kralj *et al.*, 2002, Tieking *et al.*, 2003, van Geel-Schutten *et al.*, 1998). In the draft genome sequence of *Oenococcus oeni* a glucansucrase gene identical to a dextransucrase gene (*dsrD*) from *Leuconostoc mesenteroides* Lcc4 can be found (http://www.jgi.doe.gov/JGI_microbial/html/index.html). All GTFs from lactic acid bacteria share a common structure and are composed of four distinct domains: their N-terminal end starts with (i) a signal peptide, followed by (ii) a highly variable stretch, (iii) a highly conserved catalytic or sucrose binding domain and (iv) a C-terminal glucan binding domain, composed of a series of tandem repeats (Monchois *et al.*, 1999d) (Fig. 2). The glucansucrase from *Ln. mesenteroides* NRRL-B1299 (DSRE), synthesizing a dextran with high amounts of α-(1→2) branch linkages, has an additional C-terminally located catalytic domain (Bozonnet *et al.*, 2002).

The signal peptide and variable domain of GTFs

Almost all GTFs are extracellular enzymes and the N-terminus of these enzymes contains a signal peptide for protein secretion (Fig. 2). The stretch of amino acids between the signal peptide and the core region of GTFs is highly variable both in composition and in length.

Repeating units could be identified in the variable domains of alternansucrase and dextransucrases from *Ln. mesenteroides* sp. (partial A-repeats; WY**Y**F**D**NN**G**YAVTL**GL** **QTI**N**G**QHL**YFD**AN**G**VQV**KG**, boldface type indicates conserved amino acids) (Janecek *et al.*, 2000), DSRT from *Leuconostoc mesenteroides* NRRL B-512F (motif T; TDD**KA**(A/T)**TT**A(A/D)**T**S, boldface type indicates conserved amino acids) (Funane *et*

al., 2000), DSRE from *Ln. mesenteroides* NRRL B-1299 (motif S; PA(A/T)**DKA**VD**TT**P(A/T)**T**, boldface type indicates conserved amino acids) (Bozonnet

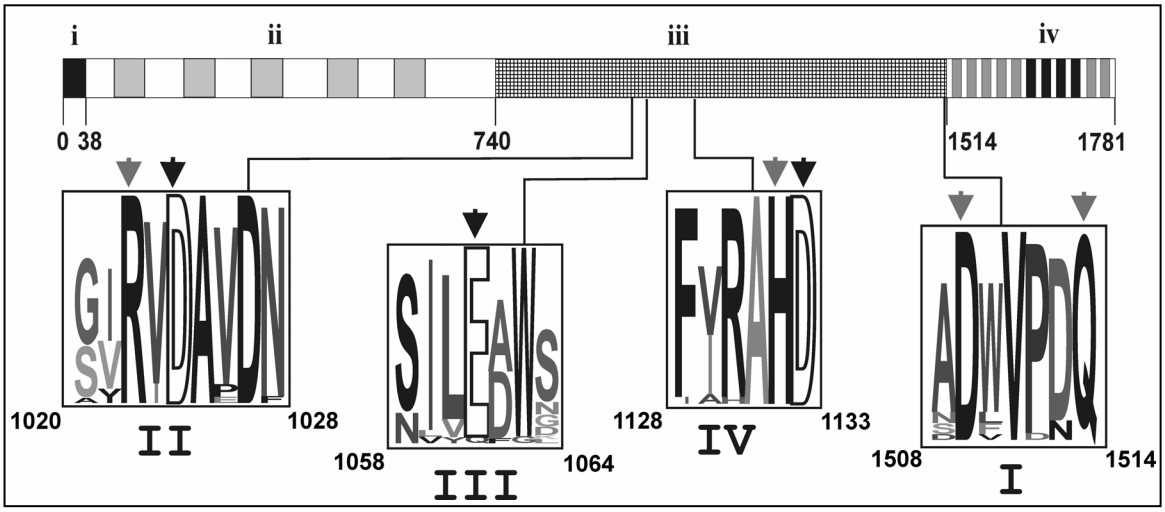


Figure 2. Schematic representation of the four different domains in GTFs (from LAB, i-iv), and the four regions with conserved amino acids (I-IV). The deduced amino acid sequence of the *Lb. reuteri* 121 glucansucrase (GTFA) was used as template (Kralj *et al.*, 2002). The four different domains shown are i) N-terminal signal sequence; ii) N-terminal variable region; iii) catalytic core; iv) C-terminal (putative) GBD. GTF sequences used are of heterologously produced GTF enzymes from lactic acid bacteria of which the distribution of glucosidic linkages in the glucans synthesized has been elucidated (Table 1). Alignments (SequenceLogo, http://www.bio.cam.ac.uk/seqlogo/) are shown of the four conserved regions (I-IV) first identified in members of the α-amylase family (GH13) (Svensson, 1994) and which can also be found in GTF enzymes (GH 70). However, as a consequence of the circular permutation, region I occurs C-terminal of region II-IV in glucansucrase enzymes (see also Fig. 3). The same seven residues fully conserved in the family GH13 are also present in the glucansucrase family (indicated with arrows), except His 327 (CGTase *B. circulans* 251 numbering), which is replaced by Gln in all GTF enzymes (Monchois *et al.*, 2000a). The three catalytic residues are indicated with white letters and black arrows.
Mutations in the catalytic nucleophile in region **II** correspond to Asp229Asn, Asp415Asn and Asp1024Asn (resulting in drastic reduced enzyme activity) of CGTase from *B. circulans* 251, GTFI from *S. mutans*, and, GTFA from *Lb. reuteri* 121, respectively (Knegtel *et al.*, 1995, Devulapalle *et al.*, 1997, Kralj *et al.*, 2004d). Mutations in the acid/base catalyst in region **III** correspond to Glu257Gln, Glu453Gln and Glu1061Gln (resulting in drastic reduced enzyme activity) from *B. circulans* 251 (Knegtel *et al.*, 1995) GTFI from *S. mutans* (Devulapalle *et al.*, 1997) and GTFA from *Lb. reuteri* 121 (Kralj *et al.*, 2004d), respectively. Mutations in the transition state stabilizer in region **IV** correspond to Asp328Asn, Asp526Asn, Asp1133Asn and Asp393Asn (resulting in drastic reduced enzyme activity) from *B. circulans* 251 (Knegtel *et al.*, 1995), GTFI from *S. mutans* (Devulapalle *et al.*, 1997), GTFA from *Lb. reuteri* 121 (Kralj *et al.*, 2004d) and amylosucrase from *Neisseria polysaccharea* (Sarcabal *et al.*, 2000), respectively. Region **I** corresponds to His140 in *B. circulans* 251 CGTase, involved in transition state stabilization (Nakamura *et al.*, 1993), which is replaced by Gln (Gln 1514 in GTFA of *Lb. reuteri* 121 in all GTF enzymes known (MacGregor *et al.*, 1996).

et al., 2002), GTFA from *Lb. reuteri* 121, GTFB from *Lb. reuteri* 121, GTFML1 from *Lb. reuteri* ML1, GTFBIO from *Lb. reuteri* BioGaia and GTF180 from *Lb. reuteri* 180 (RDV- repeats; R(P/N)DV-x_{12}-SGF-x_{19-22}-R(Y/F)S, x, non-conserved amino acid residue) (Fig. 2) (Kralj *et al.*, 2002, Kralj *et al.*, 2004b, Kralj *et al.*, 2004a), GTFKg3 from *Lactobacillus fermentum* Kg3 and GTFKg15 from *Lactobacillus sakei* Kg15 contain, respectively, 5 and 6 conserved and less-conserved YG-repeats, NDGYYFxxxGxxHºx(G/N)HºHºHº (x, non-conserved amino acid residue; Hº, hydrophobic amino acid residue) (Kralj *et al.*, 2004b, Giffard & Jacques, 1994). In the variable region of GTF33 from *Lactobacillus parabuchneri* 33, 9 short unique repeating units, designated "TTQ", were found. These repeats are 15 amino acids long (**TTTQN**(A/T)(P/A)**NN**(S/G)**N**(D/G)**PQS**, boldface type indicates conserved amino acids) and showed no significant similarity to any protein motif present in databases.

The function of the N-terminal variable domain (and these repeats) has remained unclear. Deletion studies of the complete N-terminal variable domain in GTFI of *Streptococcus downei* Mfe28 showed that it does not play a significant role in glucan structure determination (Monchois *et al.*, 1999a). Additional N-terminal deletions into the catalytic core of GTFI of *S. downei* Mfe28 resulted in drastic loss of enzyme activity (Monchois *et al.*, 1999c). The highly conserved motif "IN**G**QY**Y**" indicating the start of the catalytic core in GTF enzymes is absent in GTFB of *Lb. reuteri* 121. Its inactivity may be caused by the aberrant amino acid sequence at the start of its catalytic core (Kralj *et al.*, 2004b). The relatively large N-terminal variable domain of GTFA from *Lb. reuteri* 121 is important for initial activity with sucrose, but its deletion has only small effects on glucan characteristics (Kralj *et al.*, 2004d).

The catalytic domain of GTFs

Based on the amino acid sequences of the highly conserved catalytic domains, GTFs of *Leuconostoc* spp, *Streptococcus* spp., and *Lactobacillus* spp. belong to family 70 of glycoside hydrolase enzymes (GH70) (Henrissat & Bairoch, 1996). Up to know, there are no detailed structural data of the glucansucrases. However, in the absence of structural information an alternate approach has been to undertake molecular modeling studies of the enzymes. Secondary structure prediction studies of the catalytic domain show that glucansucrases possess a $(\beta/\alpha)_8$ barrel structure like glycosidases (including α-amylase, cyclodextrin glycosyltransferase (CGTase), and amylosucrase of family GH13 (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997). Most members of this family hydrolyze / synthesize α-(1→4) and α-(1→6) glucosidic linkages (van der Veen *et al.*, 2000). The $(\beta/\alpha)_8$-barrel structure motif is characterized by the presence of 8 β sheets (E1-E8) located in the core of the protein alternated with 8 α-helices (H1-H8) located at the surface of the protein. The $(\beta/\alpha)_8$-barrel of glucansucrases is circularly permuted. Whereas the barrel domains in α-amylases and related amylolytic enzymes begin with a

β-strand, the $(\beta/\alpha)_8$-barrel in glucansucrases starts with an α-helix and ends with a β-strand equivalent to α-helix 3 and β-strand 3, respectively, in the α-amylases (MacGregor *et al.*, 1996) (Fig. 3). The secondary structure predictions indicating the presence of a $(\beta/\alpha)_8$-barrel structure in glucansucrases was supported by circular dichroism experiments (Monchois *et al.*, 1999b). The four conserved regions (I-IV) identified in members of family GH13 (Svensson, 1994) can also be found in glucansucrases. However, as a consequence of the circular permutation, region I occurs C-terminal of region II-IV in

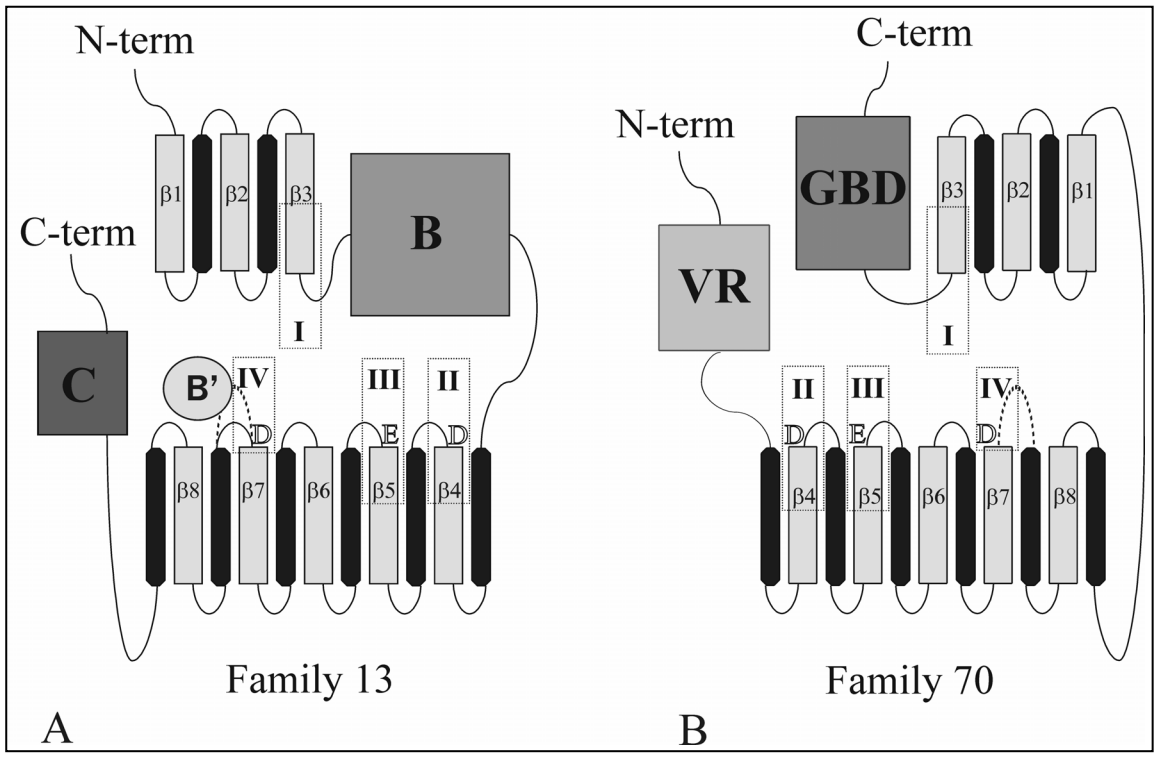


Figure 3. Topology diagrams of a family GH13 enzyme (α-amylase; A), and of glucansucrases of family GH70 (B). The catalytic domain of α-amylases has a $(\beta/\alpha)_8$ barrel structure, starting with β-strand 1 and ending with α-helix 8. The B domain is located between β-strand 3 and α-helix 3. Glucansucrases have a circularly permuted $(\beta/\alpha)_8$ barrel structure (MacGregor *et al.*, 1996), which starts with α-helix 3 (α-amylase order) and ends with β-strand 3. Between α-helix 8 and β-strand 1, a large stretch with unknown function is located. The locations of the four conserved regions (I-IV) in family GH13 (and family GH70) are indicated with dashed boxes. Amyloscurase has a domain loop (B'–domain; important for polymerizing activity; indicated with a dashed line and circle) consisting of approximately 60 amino acid residues which is located after β-strand 7 (Skov *et al.*, 2001, Skov *et al.*, 2002), immediately after the two catalytically important His392 and Asp393 residues located in conserved region IV (Sarcabal *et al.*, 2000) (Fig. 3). Glucansucrases also contain an additional "loop" (~45 amino acids; indicated with a dashed line) compared to α-amylase enzymes, which is located between β-strand 7 and α-helix 7. Conceivably, this loop is also important for polymerizing activity. The approximate sites of the three catalytic residues (D, E, and D) are indicated. B = B domain, C= C domain, GBD = Glucan Binding Domain, VR = variable region. Fig. 3A was adapted from (Nielsen & Borchert, 2000).

glucansucrase enzymes (Figs. 2, 3 and 5). The seven amino acid residues that are fully conserved in the family GH13 are also present and fully conserved in the glucansucrase family, except His327 (CGTase *Bacillus circulans* 251 numbering), which is replaced by Gln in all GTF enzymes (Glu1514, GTFA *Lb. reuteri* 121 numbering; Figs. 2 and 5) (MacGregor *et al.*, 1996, Monchois *et al.*, 2000a).

Sequence alignments, mutagenesis and X-ray structure analysis studies within family GH13, enabled the identification of several amino acid residues involved in the formation of the glucosyl-enzyme intermediates (Uitdehaag *et al.*, 1999), and / or essential for activity in family GH70 (GTF) enzymes (Monchois *et al.*, 1999d). The enzymes of family GH13 contain three amino acids with carboxyl groups important for catalysis: Asp229, Glu257 and Asp328 (CGTase *B. circulans* 251 numbering) at or near the C-terminal β-strands 4, 5 and 7 (using the structure element numbering of family GH13) (Fig. 3) (Uitdehaag *et al.*, 1999). Equivalent important residues occur invariably in GTFs: e.g. Asp415, Glu453 and Asp526 in GTFI of *S. downei* Mfe28 and Asp1024, Glu1061 and Asp1133 in GTFA of *Lb. reuteri* 121 (Figs. 2, 3 and 5) (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997, Kralj *et al.*, 2004d). Within both families the first Asp residue (catalytic nucleophile) is involved in formation of the covalent glucosyl-enzyme complex (Mooser & Iwaoka, 1989, Mooser *et al.*, 1991, MacGregor *et al.*, 1996, Uitdehaag *et al.*, 1999). However, the knowledge of primary sequences of glucansucrases alone is not sufficient for elucidation of the exact mechanism of enzyme catalysis (see below).

Efforts to obtain 3D structural information on enzymes of family GH70 have not yet succeeded. However, detailed structural information is available about amylosucrase of *Neisseria polysaccharea* (non-LAB), the only enzyme of family GH13 synthesizing a linear α-glucan from sucrose. The 3D structures of the enzyme, also in complex with sucrose, oligosaccharides and with a covalently bound glucopyranosyl moiety, resemble those of other proteins of family GH13 (Skov *et al.*, 2001, Skov *et al.*, 2002, Skov *et al.*, 2000, Mirza *et al.*, 2001, Jensen *et al.*, 2004, Uitdehaag *et al.*, 1999, Strokopytov *et al.*, 1996, Knegtel *et al.*, 1995). Amylosucrase of *N. polysaccharea* has an additional domain at the N-terminus with unknown function. In addition, the enzyme has a domain loop (B'–domain; important for polymerizing activity) consisting of approximately 60 amino acid residues which is located after β-strand 7 (Skov *et al.*, 2001, Skov *et al.*, 2002) immediately after the two catalytically important His392 and Asp393 residues located in conserved region IV (Sarcabal *et al.*, 2000) (Fig. 3). Glucansucrases also contain an additional "loop" (~45 amino acids) compared to α-amylase enzymes, which is located between β-strand 7 and α-helix 7 (Fig. 3). Conceivably, this loop is also important for polymerizing activity. In amylosucrase the Asp394 residue next to the catalytic Asp393, is part of acceptor binding site +1 and involved in the correct positioning of the glucosyl residue at this site (Albenne *et al.*, 2004). Mutagenesis of this residue resulted in changes in the product spectrum (mono and oligosaccharides) from sucrose (Albenne *et al.*, 2004).

In acarviosyltransferase (ATase) of *Actinoplanes sp.* strain SE50 the amino acid residues immediately next to the catalytic Asp328 also has interactions at subsite +1. Mutant Met329Thr in acarviosyltransferase (ATase) of *Actinoplanes sp.* strain SE50, immediately next to the catalytic Asp328 (CGTase *B. circulans* 251 numbering), also resulted in changed reaction specificity (10 × higher transferase activity on maltotetraose) (Leemhuis *et al.*, 2004).

Mutagenesis of amino acid residues in this region in different GTF enzymes resulted in altered glucan solubility and structure (see below) (Remaud-Simeon *et al.*, 2000, Shimamura *et al.*, 1994, Monchois *et al.*, 2000b, Kralj *et al.*, 2004c).

The C-terminal glucan binding domain of GTFs

Several studies have shown that the C-terminal domains of glucansucrases are involved in glucan binding. Therefore it has been designated as glucan binding domain (GBD) (Abo *et al.*, 1991, Shah & Russell, 2002, Lis *et al.*, 1995, Monchois *et al.*, 1998b, Kralj *et al.*, 2004d, Kato & Kuramitsu, 1990, Kingston *et al.*, 2002). The C-terminal domain could possibly be involved in the determination of the structure of the synthesized glucan (Vickerman *et al.*, 1996, Kralj *et al.*, 2004d). The C-terminal GBD appears to be necessary for glucansucrase activity (Nakano & Kuramitsu, 1992, Kralj *et al.*, 2004d). Examples exist, e.g. GTFI of *S. downei* Mfe28, where deletion of a large part of the C-terminal GBD had a small effect on enzyme activity (Monchois *et al.*, 1999a). Some GTF enzymes with deletions at the C-terminal end retained hydrolytic activity but glucan binding and synthesis properties had disappeared (Funane *et al.*, 2000). The precise role of this GBD domain in enzyme catalysis remains largely unknown. The GBD may be of importance for polymer chain growth. It has been suggested that the C-terminal domain could also play a facilitating role in the transfer of products from the catalytic site (Monchois *et al.*, 1998b).

The C-terminal domains of all reported glucansucrases are composed of a series of repeating units, which have been divided into four classes; A, B, C and D-repeats. Within these repeats, a common conserved stretch of amino acids, designated YG-repeat, NDGYYFxxxGxxHºx(G/N)HºHºHº (x, non-conserved amino acid residue; Hº, hydrophobic amino acid residue) can be distinguished (Giffard & Jacques, 1994). The number, class, and distribution of the repeats are specific for each enzyme. *Ln. mesenteroides* NRRL B-512F dextransucrase contains besides A and C repeats also N repeats, which have not been identified in streptococcal GTFs. These N repeats are not highly conserved but possess the main characteristics of YG-repeats (Monchois *et al.*, 1998b). Alternansucrase from *Ln. mesenteroides* NRRL B-1355 contains a single A repeat and distinct short repeats $DG(X)_4APY$ (Arguello-Morales *et al.*, 2000). GTFA from *Lb. reuteri* 121, GTFB from *Lb. reuteri* 121, GTFML1 from *Lb. reuteri* ML1, GTFBIO from *Lb. reuteri* BioGaia and GTF180 from *Lb. reuteri* 180 possess relatively

short GBDs of 134-263 amino acids (Fig. 2; average length is 400-500 amino acids in other GTFs) (Monchois *et al.*, 1999d), consisting of several conserved and less well conserved YG-repeats (Kralj *et al.*, 2002, Kralj *et al.*, 2004a, Kralj *et al.*, 2004b). The putative GBDs of GTFKg15 from *Lb. sakei* Kg15, GTFKg3 from *Lb. fermentum* Kg3 and GTF33 from *Lb. parabuchneri* 33 contained a varying number of conserved and less well conserved YG repeating units and no A, B, C or D repeats could be identified. GTF33 contains besides the 17 YG-repeats, two unique repeating units designated "KYQ" (49 amino acids, AVK(T/A)A(K/Q)(A/T)(Q/K)(L/V)(A/N)K(T/A)KAQ(I/V)(A/T)KYQKAL KKAKTTKAK(A/T)QARK(S/N)LKKA(E/N(T/S)S(F/L)(S/T)KA) that showed no significant similarity to any protein motif present in databases. GTFKg15 possesses an additional stretch at the end of its putative GBD, which shows similarity to part of a putative extracellular matrix binding protein from *Streptococcus pyogenes* M1 (AE006525; 44% similarity and 56% identity within 75 amino acids) (Kralj *et al.*, 2004b).

Catalytic mechanism of glucansucrases

Glucan polymer synthesis by glucansucrase enzymes proceeds in a processive manner, intermediate oligosaccharides can not be detected and polysaccharides of high molecular weight are obtained at early reaction times (Bovey, 1959, Ebert & Schenk, 1968, Tsuchiya *et al.*, 1953). Two alternative mechanisms have been proposed for the glucan chain growth (Fig. 4): **a**) *Non-reducing end elongation*; this mechanism involves the presence of one site (an aspartate or glutamate) acting as a nucleophilic group and another residue acting as a proton donor, **b**) *Reducing end elongation*; according to this mechanism, the reaction occurs in two steps involving two sucrose binding sites (nucleophiles) (Robyt *et al.*, 1974, Robyt, 1996, Monchois *et al.*, 1999d)

Amylosucrase is expected to use the same double-displacement mechanism as other family 13 members (Uitdehaag *et al.*, 1999, Albenne *et al.*, 2004, Jensen *et al.*, 2004), in which a covalent glucosyl-enzyme complex intermediate is formed. In a subsequent step this glucosyl moiety is transferred onto a water molecule (hydrolysis; main reaction catalyzed by α-amylases) or onto a hydroxyl group of a sugar acceptor (transglucosylation reaction). Both steps are suggested to proceed via oxocarbonium ion-like transition states. Polymer formation by amylosucrase proceeds in a nonprocessive way (release of the polymer chain after each glucosyl residue transfer) by elongation of the glucan chain at the non-reducing end. (Albenne *et al.*, 2004).

Glucansucrases of family 70 elongate oligosaccharides at the non-reducing end (Arguello Morales *et al.*, 2001, Dols *et al.*, 1997, Mukasa *et al.*, 2000, Monchois *et al.*, 2000a, Kralj *et al.*, 2004a) possibly using the same mechanism as amylosucrase / family 13 enzymes. Glucan elongation by glucansucrases possibly also takes place at the non-reducing end. This hypothesis is supported by the fact that up to now only one site capable of making a covalent bond with the glucose moiety originating from the

breakdown of sucrose has been clearly identified (Mooser *et al.*, 1991). Nevertheless, further analysis of polymer formation by glucansucrases is required to elucidate the exact reaction mechanism and to allow comparison with the mode of action of amylosucrase.

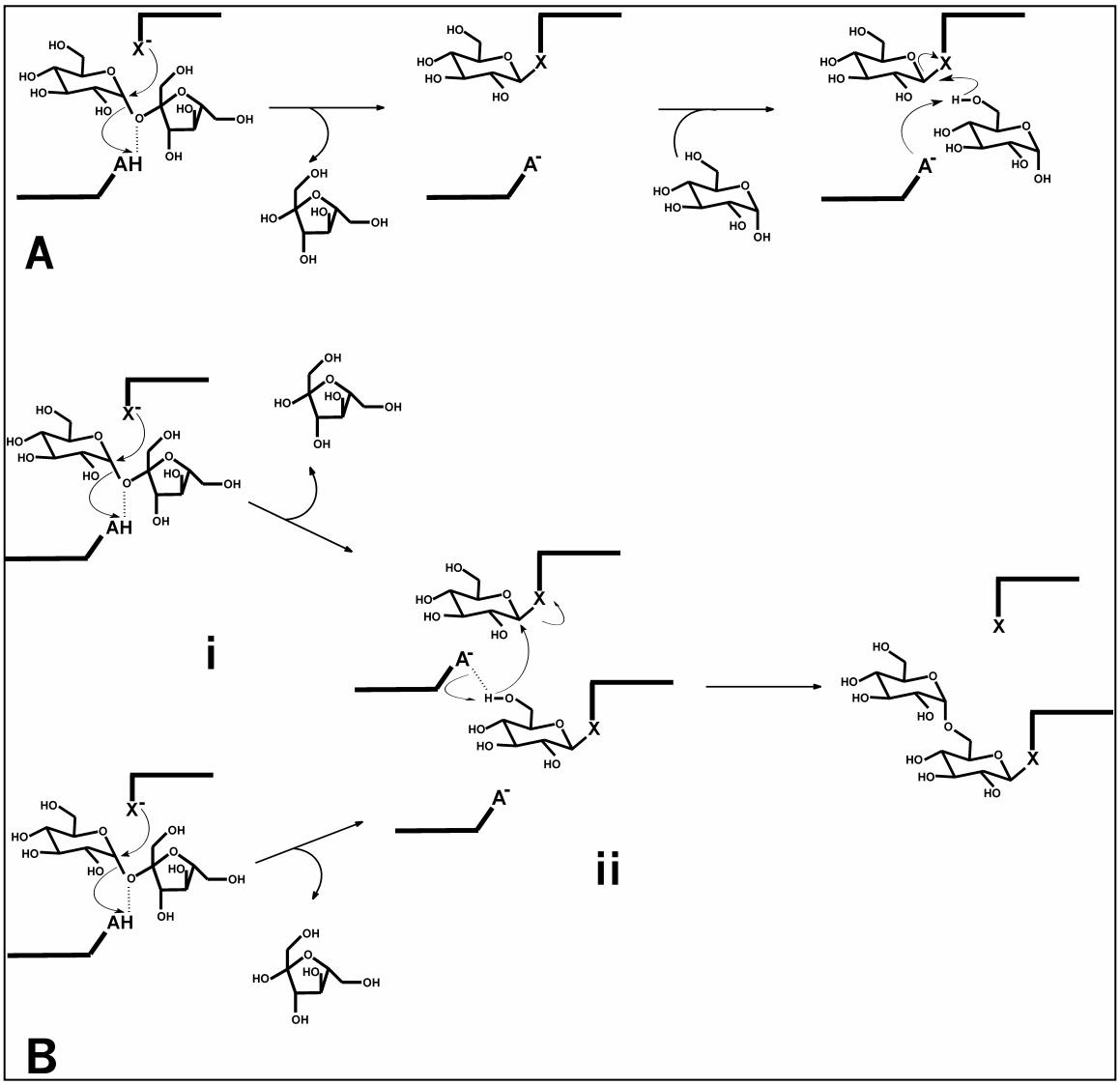


Figure 4. The two reaction mechanisms postulated for glucansucrase enzymes.
(A) Non-reducing end elongation: only one covalent glucosyl-enzyme intermediate is involved. The glucan chain grows by successive insertions of glucose units between the catalytic site of the enzyme and the reducing end of the glucan polymer (Monchois *et al.*, 1999d). **(B)** Reducing end elongation: according to this mechanism, the reaction occurs in two steps involving two sucrose binding sites (nucleophiles, presumably carboxylate anions): (i) the nucleophilic sites attack two sucrose molecules to give two covalent glucosyl-enzyme intermediates, and (ii) the C-6 hydroxyl of one of the glucosyl intermediates makes a nucleophilic attack onto C-1 of the other glucosyl intermediate to form an α-(1→6) glucosidic linkage and an isomaltosyl intermediate. The newly released nucleophilic site attacks another sucrose molecule to give a new glucosyl-enzyme intermediate. This symmetrical and alternative role of the two sucrose binding sites results in growth of the glucan chain by its reducing end (Robyt, 1996). X, nucleophilic group; A, proton donor group.

Oligosaccharide synthesis by glucansucrases

Koepsel et al. (Koepsell *et al.*, 1953) observed that in the presence of sucrose and acceptor molecules such as maltose, isomaltose, and O-α-methylglucoside, glucansucrase enzymes shifted from glucan synthesis towards the production of oligosaccharides (acceptor reaction). Most acceptor studies are performed using saccharides (Fu & Robyt, 1991, Dols-Lafargue *et al.*, 2001, Arguello Morales *et al.*, 2001, Dols *et al.*, 1997, Kralj *et al.*, 2004a, Kralj *et al.*, 2004d) or saccharide derivatives as acceptor substrates (Demuth *et al.*, 2002, Richard *et al.*, 2003, Cote & Dunlap, 2003). Also aromatic compounds (e.g. catechine) and salicyl alcohol have been shown to act as acceptor substrates (Meulenbeld & Hartmans, 2000, Yoon *et al.*, 2004). Acceptors can be divided in two main classes: i) strong acceptors (e.g. maltose) which inhibit the synthesis of glucan polymer and ii) weak acceptors (e.g. fructose). The reaction with fructose becomes important at the end of the reaction when (leucrose, α-D-glucopyranosyl-(1→5)-β-D-fructofuranoside, the only acceptor product synthesized from fructose) is formed.

Dextransucrase from *Ln. mesenteroides* NRRL B-512F uses leucrose and sucrose itself not as acceptor, whereas the sucrose analogues isomaltulose (α-D-glucopyranosyl-(1→6)-β-D-fructofuranoside) and trehalulose (α-D-glucopyranosyl-(1→1)-β-D-fructofuranoside), and turanose (α-D-glucopyranosyl-(1→3)-β-D-fructofuranoside) and maltulose (α-D-glucopyranosyl-(1→4)-β-D-fructofuranoside) can be classified as strong acceptors and moderate acceptors, respectively (Demuth *et al.*, 2002).

GLUCANSUCRASES OF LACTIC ACID BACTERIA

A large number of glucansucrases genes have been sequenced, for an overview consult (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). The distribution of glucosidic linkages has been elucidated for the glucans synthesized by heterologously produced GTF enzymes from (i) 13 GTFs of seven *Streptococcus* strains (Monchois *et al.*, 1999d, Hanada *et al.*, 2002, Konishi *et al.*, 1999), (ii) 7 GTFs of four *Leuconostoc* strains (Monchois *et al.*, 1999d, Bozonnet *et al.*, 2002, Neubauer *et al.*, 2003, Funane *et al.*, 2001, Arguello-Morales *et al.*, 2000), and (iii) 7 GTFs of seven *Lb. reuteri* strains (Kralj *et al.*, 2002, Kralj *et al.*, 2004a, Kralj *et al.*, 2004b) (Table 1).

Only *gtf* genes encoding either dextran- or mutansucrase enzymes have been characterized in the genus *Streptococcus* (Monchois *et al.*, 1999d, Hanada *et al.*, 2002, Konishi *et al.*, 1999). *Leuconostoc* strains carry *gtf* genes encoding mainly dextransucrase enzymes, but also one alternansucrase encoding gene, and one gene encoding a glucansucrase synthesizing large amounts of α-(1→2) branch linkages has been characterized (Arguello-Morales *et al.*, 2000, Bozonnet *et al.*, 2002, Monchois *et al.*, 1999d). *Lactobacillus* strains contain *gtf* genes encoding mainly dextransucrase enzymes. However, also two reuteran producing lactobacilli have been identified and one

Lactobacillus strain which synthesized a highly branched mutan (Kralj *et al.*, 2002, Kralj *et al.*, 2004a, Kralj *et al.*, 2004b) (Table 1).

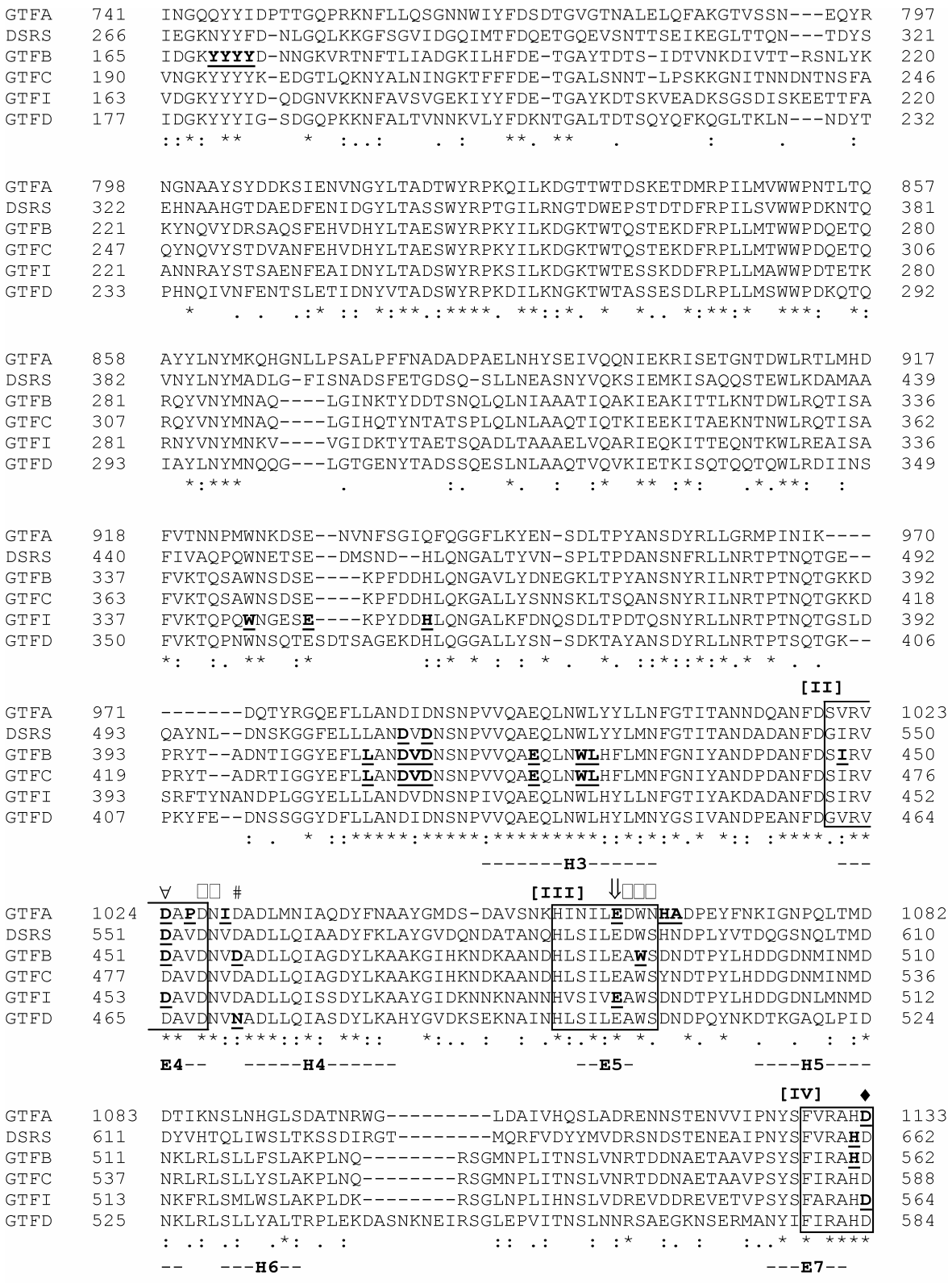
Table 1. Main characteristics of glucansucrases and glucans synthesized by heterologously produced GTF enzymes from lactic acid bacteria. * method used in linkage type analysis M = methylation; ^{13}C = ^{13}C – NMR; ^{1}H = ^{1}H –NMR

Glucan Strain	**Gene**	**Glucan linkage type distribution (%)**				**Size (aa)**	**Method***	**References**
Reuteran		α-(1→)	**α-(1→4)**	α-(1→6)	α-(1→4,6)			
Lb. reuteri 121	*gtfA*	11	46	26	17	1781	M/^{1}H	[100,105]
Lb. reuteri BioGaia	*gtfBio*	9	69	11	13	1781	M/^{1}H	[102]
Dextran		α-(1→)	α-(1→3)	**α-(1→6)**	α-(1→3,6)			
Lb. reuteri 180	*gtf180*	10	26	51	13	1772	M	[103]
Lb. sakei Kg15	*gtfKg15*	4	9	86	9	1561	M	[103]
Lb. fermentum Kg3	*gtfKg3*	3	7	89	7	1595	M	[103]
Lb. parabuchneri 33	*gtf33*	6	9	75	9	1463	M	[103]
Ln. mesenteroides NRRL B-1299	*dsrA*		15	85		1290	^{13}C	[124]
Ln. mesenteroides NRRL B-1299	*dsrB*		5	95		1508	^{13}C	[126]
Ln. mesenteroides NRRL B-512F	*dsrS*		5	95		1527	^{13}C	[125,161]
Ln. mesenteroides NRRL B-512F	*dsrT5*		40	50		1499	^{13}C	[58,59]
Ln. mesenteroides Lcc4	*dsrD*			mainly		1527	^{13}C	[144]
S. mutans GS 5	*gtfD*		15	70	15	1430	M	[74,83,178]
S. downei Mfe28	*gtfS*		90			1365	^{13}C	[66]
S. sobrinus OMZ176	*gtfT*	6	16	73	5	1468	M	[73]
S. sobrinus B13N	*gtfU*	44		25	31	1554	^{13}C	[75]
S. salivarius ATCC 25975	*gtfK*			100		1599	^{13}C	[184]
S. salivarius ATCC 25975	*gtfM*			95		1577	^{13}C	[184]
S. gordonii CH1	*gtfG*		25	75		1577	^{13}C	[69,216,217]
Mutan		α-(1→)	**α-(1→3)**	α-(1→6)	α-(1→3,6)			
Lb. reuteri ML1	*gtfML1*	18	47	26	13	1772	M	[103]
S. mutans GS 5	*gtfB*		88	7	5	1475	M	[56,179]
S. mutans GS 5	*gtfC*		86	8	7	1375	M	[56,202]
S. downei Mfe28	*gtfI*	0.5	88	2	0.5	1597	^{13}C	[166]
S. sobrinus 6715	*gtfIa*		mainly			1592	^{13}C	[98]
S. salivarius ATCC 25975	*gtfJ*		90			1517	^{13}C	[184]
Glucan								
Ln. mesenteroides NRRL B-1355	*asr*	Alternating α-(1→3)/α-(1→6) linkages (**Alternan**)				2057	M/^{13}C	[7,31]
Ln. mesenteroides NRRL B-1299	*dsrE*	**α-(1→2,6)** & α-(1→6)				2835	M/^{13}C	[17,175]
S. salivarius ATCC 25975	*gtfL*	Equal amounts α-(1→3)/α-(1→6) linkages				1449	^{13}C	[184]

GLUCANSUCRASE SITE-DIRECTED MUTANTS

Identification of the catalytic site

The three amino acid residues involved in catalysis (see above) occur invariably in GTFs. These are Asp415, Glu453 and Asp526 in GTFI of *S. downei* Mfe28 and Asp1024, Glu1061 and Asp1133 in GTFA of *Lb. reuteri* 121 (Figs. 2 and 5) (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997, Kralj *et al.*, 2004d). The importance of the first Asp residue has also been shown for other GTFs by site-directed mutagenesis experiments (Kato *et al.*, 1992, Monchois *et al.*, 1997) (Fig. 5).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DSRS 266 IEGKNYYFD-NLGQLKKGFSGVIDGQIMTFDQETGQEVSNTTSEIKEGLTTQN---TDYS 321
GTFB 165 IDGKYYYYD-NNGKVRTNFTLIADGKILHFDE-TGAYTDTS-IDTVNKDIVTT-RSNLYK 220
GTFC 190 VNGKYYYYK-EDGTLQKNYALNINGKTFFFDE-TGALSNNT-LPSKKGNITNNDNTNSFA 246
GTFI 163 VDGKYYYYD-QDGNVKKNFAVSVGEKIYYFDE-TGAYKDTSKVEADKSGSDISKEETTFA 220
GTFD 177 IDGKYYYIG-SDGQPKKNFALTVNNKVLYFDKNTGALTDTSQYQFKQGLTKLN---NDYT 232
GTFA 798 NGNAAYSYDDKSIENVNGYLTADTWYRPKQILKDGTTWTDSKETDMRPILMVWWPNTLTQ 857
DSRS 322 EHNAAHGTDAEDFENIDGYLTASSWYRPTGILRNGTDWEPSTDTDFRPILSVWWPDKNTQ 381
GTFB 221 KYNQVYDRSAQSFEHVDHYLTAESWYRPKYILKDGKTWTQSTEKDFRPLLMTWWPDQETQ 280
GTFC 247 QYNQVYSTDVANFEHVDHYLTAESWYRPKYILKDGKTWTQSTEKDFRPLLMTWWPDQETQ 306
GTFI 221 ANNRAYSTSAENFEAIDNYLTADSWYRPKSILKDGKTWTESSKDDFRPLLMAWWPDTETK 280
GTFD 233 PHNQIVNFENTSLETIDNYVTADSWYRPKDILKNGKTWTASSESDLRPLLMSWWPDKQTQ 292
GTFA 858 AYYLNYMKQHGNLLPSALPFFNADADPAELNHYSEIVQQNIEKRISETGNTDWLRTLMHD 917
DSRS 382 VNYLNYMADLG-FISNADSFETGDSQ-SLLNEASNYVQKSIEMKISAQQSTEWLKDAMAA 439
GTFB 281 RQYVNYMNAQ----LGINKTYDDTSNQLQLNIAAATIQAKIEAKITTLKNTDWLRQTISA 336
GTFC 307 RQYVNYMNAQ----LGIHQTYNTATSPLQLNLAAQTIQTKIEEKITAEKNTNWLRQTISA 362
GTFI 281 RNYVNYMNKV----VGIDKTYTAETSQADLTAAAELVQARIEQKITTEQNTKWLREAISA 336
GTFD 293 IAYLNYMNQQG---LGTGENYTADSSQESLNLAAQTVQVKIETKISQTQQTQWLRDIINS 349
GTFA 918 FVTNNPMWNKDSE--NVNFSGIQFQGGFLKYEN-SDLTPYANSDYRLLGRMPINIK---- 970
DSRS 440 FIVAQPQWNETSE--DMSND--HLQNGALTYVN-SPLTPDANSNFRLLNRTPTNQTGE-- 492
GTFB 337 FVKTQSAWNSDSE----KPFDDHLQNGAVLYDNEGKLTPYANSNYRILNRTPTNQTGKKD 392
GTFC 363 FVKTQSAWNSDSE----KPFDDHLQKGALLYSNNSKLTSQANSNYRILNRTPTNQTGKKD 418
GTFI 337 FVKTQPQWNGESE----KPYDDHLQNGALKFDNQSDLTPDTQSNYRLLNRTPTNQTGSLD 392
GTFD 350 FVKTQPNWNSQTESDTSAGEKDHLQGGALLYSN-SDKTAYANSDYRLLNRTPTSQTGK-- 406
[II]
GTFA 971 -------DQTYRGQEFLLANDIDNSNPVVQAEQLNWLYYLLNFGTITANNDQANFDSVRV 1023
DSRS 493 QAYNL--DNSKGGFELLLANDVDNSNPVVQAEQLNWLYYLMNFGTITANDADANFDGIRV 550
GTFB 393 PRYT--ADNTIGGYEFLLANDVDNSNPVVQAEQLNWLHFLMNFGNIYANDPDANFDSIRV 450
GTFC 419 PRYT--ADRTIGGYEFLLANDVDNSNPVVQAEQLNWLHFLMNFGNIYANDPDANFDSIRV 476
GTFI 393 SRFTYNANDPLGGYELLLANDVDNSNPIVQAEQLNWLHYLLNFGTIYAKDADANFDSIRV 452
GTFD 407 PKYFE--DNSSGGYDFLLANDIDNSNPVVQAEQLNWLHYLMNYGSIVANDPEANFDGVRV 464
-------H3------
[III]
GTFA 1024 DAPDNIDADLMNIAQDYFNAAYGMDS-DAVSNKHINILEDWNHADPEYFNKIGNPQLTMD 1082
DSRS 551 DAVDNVDADLLQIAADYFKLAYGVDQNDATANQHLSILEDWSHNDPLYVTDQGSNQLTMD 610
GTFB 451 DAVDNVDADLLQIAGDYLKAAKGIHKNDKAANDHLSILEAWSDNDTPYLHDDGDNMINMD 510
GTFC 477 DAVDNVDADLLQIAGDYLKAAKGIHKNDKAANDHLSILEAWSYNDTPYLHDDGDNMINMD 536
GTFI 453 DAVDNVDADLLQISSDYLKAAYGIDKNNKNANNHVSIVEAWSDNDTPYLHDDGDNLMNMD 512
GTFD 465 DAVDNVNADLLQIASDYLKAHYGVDKSEKNAINHLSILEAWSDNDPQYNKDTKGAQLPID 524
E4-- -----H4------ --E5- ----H5----
[IV]
GTFA 1083 DTIKNSLNHGLSDATNRWG---------LDAIVHQSLADRENNSTENVVIPNYSFVRAHD 1133
DSRS 611 DYVHTQLIWSLTKSSDIRGT--------MQRFVDYYMVDRSNDSTENEAIPNYSFVRAHD 662
GTFB 511 NKLRLSLLFSLAKPLNQ--------RSGMNPLITNSLVNRTDDNAETAAVPSYSFIRAHD 562
GTFC 537 NRLRLSLLYSLAKPLNQ--------RSGMNPLITNSLVNRTDDNAETAAVPSYSFIRAHD 588
GTFI 513 NKFRLSMLWSLAKPLDK--------RSGLNPLIHNSLVDREVDDREVETVPSYSFARAHD 564
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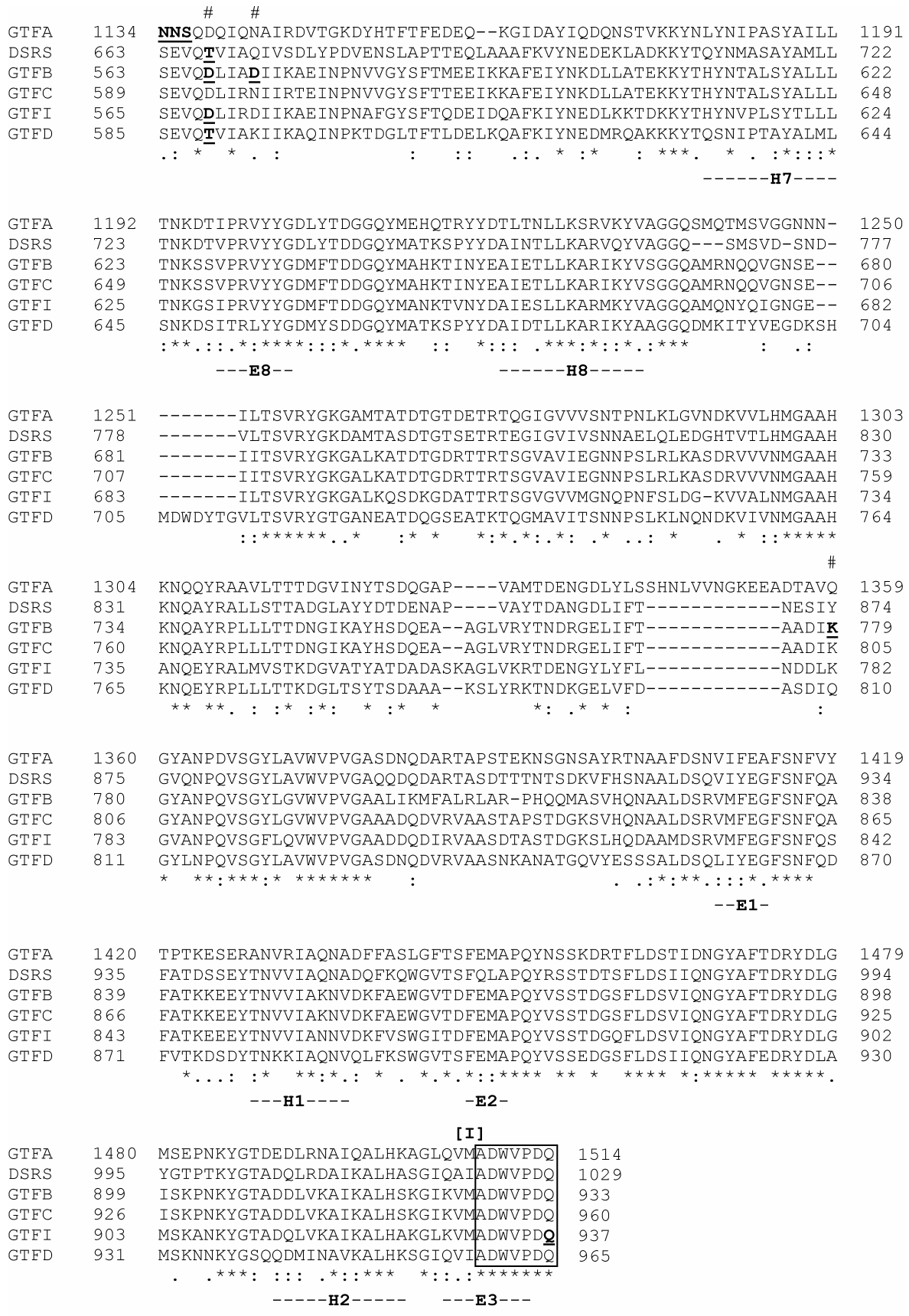
--- ---H6-- ---E7--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GTFB 563 SEVQDLIADIIKAEINPNVVGYSFTMEEIKKAFEIYNKDLLATEKKYTHYNTALSYALLL 622
GTFC 589 SEVQDLIRNIIRTEINPNVVGYSFTTEEIKKAFEIYNKDLLATEKKYTHYNTALSYALLL 648
GTFI 565 SEVQDLIRDIIKAEINPNAFGYSFTQDEIDQAFKIYNEDLKKTDKKYTHYNVPLSYTLLL 624
GTFD 585 SEVQTVIAKIIKAQINPKTDGLTFTLDELKQAFKIYNEDMRQAKKKYTQSNIPTAYALML 644
H7
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DSRS 723 TNKDTVPRVYYGDLYTDDGQYMATKSPYYDAINTLLKARVQYVAGGQ---SMSVD-SND- 777
GTFB 623 TNKSSVPRVYYGDMFTDDGQYMAHKTINYEAIETLLKARIKYVSGGQAMRNQQVGNSE-- 680
GTFC 649 TNKSSVPRVYYGDMFTDDGQYMAHKTINYEAIETLLKARIKYVSGGQAMRNQQVGNSE-- 706
GTFI 625 TNKGSIPRVYYGDMFTDDGQYMANKTVNYDAIESLLKARMKYVAGGQAMQNYQIGNGE-- 682
GTFD 645 SNKDSITRLYYGDMYSDDGQYMATKSPYYDAIDTLLKARIKYAAGGQDMKITYVEGDKSH 704
E8
H8
GTFA 1251 -------ILTSVRYGKGAMTATDTGTDETRTQGIGVVVSNTPNLKLGVNDKVVLHMGAAH 1303
DSRS 778 -------VLTSVRYGKDAMTASDTGTSETRTEGIGVIVSNNAELQLEDGHTVTLHMGAAH 830
GTFB 681 -------IITSVRYGKGALKATDTGDRTTRTSGVAVIEGNNPSLRLKASDRVVVNMGAAH 733
GTFC 707 -------IITSVRYGKGALKATDTGDRTTRTSGVAVIEGNNPSLRLKASDRVVVNMGAAH 759
GTFI 683 -------ILTSVRYGKGALKQSDKGDATTRTSGVGVVMGNQPNFSLDG-KVVALNMGAAH 734
GTFD 705 MDWDYTGVLTSVRYGTGANEATDQGSEATKTQGMAVITSNNPSLKLNQNDKVIVNMGAAH 764
GTFA 1304 KNQQYRAAVLTTTDGVINYTSDQGAP----VAMTDENGDLYLSSHNLVVNGKEEADTAVQ 1359
DSRS 831 KNQAYRALLSTTADGLAYYDTDENAP----VAYTDANGDLIFT------------NESIY 874
GTFB 734 KNQAYRPLLLTTDNGIKAYHSDQEA--AGLVRYTNDRGELIFT------------AADIK 779
GTFC 760 KNQAYRPLLLTTDNGIKAYHSDQEA--AGLVRYTNDRGELIFT------------AADIK 805
GTFI 735 ANQEYRALMVSTKDGVATYATDADASKAGLVKRTDENGYLYFL------------NDDLK 782
GTFD 765 KNQEYRPLLLTTKDGLTSYTSDAAA--KSLYRKTNDKGELVFD------------ASDIQ 810
GTFA 1360 GYANPDVSGYLAVWVPVGASDNQDARTAPSTEKNSGNSAYRTNAAFDSNVIFEAFSNFVY 1419
DSRS 875 GVQNPQVSGYLAVWVPVGAQQDQDARTASDTTTNTSDKVFHSNAALDSQVIYEGFSNFQA 934
GTFB 780 GYANPQVSGYLGVWVPVGAALIKMFALRLAR-PHQQMASVHQNAALDSRVMFEGFSNFQA 838
GTFC 806 GYANPQVSGYLGVWVPVGAAADQDVRVAASTAPSTDGKSVHQNAALDSRVMFEGFSNFQA 865
GTFI 783 GVANPQVSGFLQVWVPVGAADDQDIRVAASDTASTDGKSLHQDAAMDSRVMFEGFSNFQS 842
GTFD 811 GYLNPQVSGYLAVWVPVGASDNQDVRVAASNKANATGQVYESSSALDSQLIYEGFSNFQD 870
E1
GTFA 1420 TPTKESERANVRIAQNADFFASLGFTSFEMAPQYNSSKDRTFLDSTIDNGYAFTDRYDLG 1479
DSRS 935 FATDSSEYTNVVIAQNADQFKQWGVTSFQLAPQYRSSTDTSFLDSIIQNGYAFTDRYDLG 994
GTFB 839 FATKKEEYTNVVIAKNVDKFAEWGVTDFEMAPQYVSSTDGSFLDSVIQNGYAFTDRYDLG 898
GTFC 866 FATKKEEYTNVVIAKNVDKFAEWGVTDFEMAPQYVSSTDGSFLDSVIQNGYAFTDRYDLG 925
GTFI 843 FATKEEEYTNVVIANNVDKFVSWGITDFEMAPQYVSSTDGQFLDSVIQNGYAFTDRYDLG 902
GTFD 871 FVTKDSDYTNKKIAQNVQLFKSWGVTSFEMAPQYVSSEDGSFLDSIIQNGYAFEDRYDLA 930
H1
E2
[I]
GTFA 1480 MSEPNKYGTDEDLRNAIQALHKAGLQVMADWVPDQ 1514
DSRS 995 YGTPTKYGTADQLRDAIKALHASGIQAIADWVPDQ 1029
GTFB 899 ISKPNKYGTADDLVKAIKALHSKGIKVMADWVPDQ 933
GTFC 926 ISKPNKYGTADDLVKAIKALHSKGIKVMADWVPDQ 960
GTFI 903 MSKANKYGTADQLVKAIKALHAKGLKVMADWVPDQ 937
GTFD 931 MSKNNKYGSQQDMINAVKALHKSGIQVIADWVPDQ 965
H2
E3

Figure 5. Sequence alignment of the conserved catalytic core of glucansucrases, GTFA of *Lb. reuteri* 121 (Kralj *et al.*, 2002), DSRS of *Ln. mesenteroides* NRRL B-512F (Monchois *et al.*, 1997), GTFB of *S. mutans* GS5 (Shiroza *et al.*, 1987), GTFC of *S. mutans* GS5 (Ueda *et al.*, 1988), GTFI of *S. downei* Mfe28 (Russell *et al.*, 1987) and GTFD of *S. mutans* GS5 (Honda *et al.*, 1990), indicating amino acid residues which have been subjected to site-directed mutagenesis studies (Chia *et al.*, 1998, Monchois *et al.*, 1999c, Monchois *et al.*, 2000a, Monchois *et al.*, 1997, Devulapalle *et al.*, 1997, Monchois *et al.*, 2000b, Remaud-Simeon *et al.*, 2000, Tsumori *et al.*, 1997, Kralj *et al.*, 2004d, Kralj *et al.*, 2004c).
***** , identical residue; **:** , highly conserved residue; **.** , conserved residue; ∀, putative nucleophile (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); ⇓, putative acid/base catalyst (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); ♦, putative residue stabilizing the transition state (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); □, putative acceptor binding / glycosyl transfer sites (MacGregor *et al.*, 1996); #, residues involved in glucan solubility and structure determination [α-(1→3)/α-(1→6)] as shown separately for different glucansucrases, mutated amino acids shown in bold face and underlined (Shimamura *et al.*, 1994, Remaud-Simeon *et al.*, 2000, Monchois *et al.*, 2000a); **aa**, amino acid residues that have been mutated; -**Ex**-, localization of β-strands; -**Hx**-, localization of α-helices according to (MacGregor *et al.*, 1996). The four conserved regions (I-IV) identified in members of family GH13 (Svensson, 1994) can also be found in glucansucrases and are depicted boxed. Sequence of peptides to generate antibodies are lightly shaded (Dertzbaugh & Macrina, 1990, Chia *et al.*, 1993, Chia *et al.*, 1997).

Members of the α-amylase superfamily contain two histidine residues involved in activity and proposed to be involved in stabilization of the transition state (Nakamura *et al.*, 1993, MacGregor *et al.*, 1996). One of those present in family GH13 (His327 in *B. circulans* 251 CGTase) is conserved in GTF enzymes (His1132 in *Lb. reuteri* 121 GTFA; Figs. 2 and 5). Its mutation (His661Arg in DSRS of *Ln. mesenteroides* NRRL B-512F and His561Gly in GTFB of *Streptococcus mutans* GS5) resulted in very low enzyme activity (Tsumori *et al.*, 1997, Monchois *et al.*, 1997) (Fig. 5). This residue thus may have a similar role in GTF enzymes. The other histidine residue (His140 in *B. circulans* 251 CGTase), involved in transition state stabilization (Nakamura *et al.*, 1993), is replaced by Gln in all GTF enzymes known (Gln1514 in *Lb. reuteri* 121 GTFA; Figs. 2 and 5) (MacGregor *et al.*, 1996, Monchois *et al.*, 2000a). Mutagenesis of this residue, mutation Gln937His in GTFI of *Streptococcus downei*, resulted in drastic but not complete loss of activity, suggesting that Gln937 plays no direct role in the cleavage of sucrose and in the formation of the covalent glucosyl-enzyme intermediate, but may also be important for transition state stabilization (Monchois *et al.*, 2000a). In CGTase, mutation of the corresponding histidine residue (His140) resulted in lowered activity and altered distribution of α-, β-, and γ-cyclodextrins produced (Nakamura *et al.*, 1993). Mutagenesis of Gln937Asn in GTFI also resulted in reduced activity and modified distribution of oligodextran and nigerooligosaccharide products (Monchois *et al.*, 2000a). For two of the seven fully conserved residues, Arg1022 and Asp 1509, *Lb. reuteri* 121 GTFA numbering, no mutagenesis data is available for GTF enzymes. However, in CGTase mutagenesis of

the corresponding residues (Arg227 and Asp135, CGTase *B. circulans* 251 numbering) resulted in a drastic decrease of activity (Leemhuis *et al.*, 2003).

Regions influencing the structure of the glucan and oligosaccharide products

GTFA derivatives containing mutation P1026V (located in conserved region II) showed a clear change in oligosaccharide and glucan products, with an increase of α-(1→6) and a decrease of α-(1→4) glucosidic linkages (Kralj *et al.*, 2004c). Mutations in the tripeptide immediately following the (putative) transition state stabilizing residue in GTFA of *Lb. reuteri* 121 (N1134S:N1135E:S1136V) resulted in a drastic increase in α-(1→6) linkages (~40%) and a decrease in α-(1→4) linkages (~40%) in the polymer synthesized compared to wild type (Kralj *et al.*, 2004c). A quintuple mutant was constructed, by combination of the triple amino acid mutant (N1134S:N1135E:S1136V) near region IV, with a double mutant (P1026V:I1029V) located in/near region II (Fig. 5), which resulted in an even further enhanced ratio of α-(1→6)/α-(1→4) glucosidic linkages in its oligosaccharide and glucan products (Kralj *et al.*, 2004c).

Mutation T667R, located C-terminal of conserved region IV, in DSRS of *Ln. mesenteroides* NRRL B-512F resulted in 8% more α-(1→3) linkages in the dextran product (Remaud-Simeon *et al.*, 2000).

Identification of other important regions

In different GTF enzymes, mutations in an amino acid residue, located five amino acids behind the catalytic Asp1133 (similar to T667R in DSRS of *Ln. mesenteroides* NRRL B-512F), resulted in a shift in glucan (in)solubility (Fig. 5). Mutation T589E in GTFD of *S. mutans* GS5 lowered the amount of soluble glucan synthesized from 86% to 2% (Shimamura *et al.*, 1994). The reverse shift, from a completely insoluble glucan to more soluble glucan synthesis, was observed when the similar amino acid residue was mutated in GTFB (mutant D567T) of *S. mutans* GS5 (increase in soluble glucan from 0% to 24 %) and in GTFI (mutant D569T) of *S. downei* Mfe28 (Shimamura *et al.*, 1994, Monchois *et al.*, 2000b).

Mutation of I448V, located in conserved region II, in GTFB of *S. mutans* GS5 did not change the insolubility. Other amino acid residues influencing the (in)solubility of the glucan are: D457N (C-terminal of conserved region II), D571K (C-terminal of conserved region IV), K779Q and K1014T in GTFB of *S. mutans* GS5 and N471D (C-terminal of conserved region IV) in GTFD of *S. mutans* GS5, resulting in an increase of soluble glucan synthesis (from 0 to 37%), (from 0 to 18%), (from 0 to3 %) and (from 0 to14 %) and insoluble glucan synthesis (from 14 to 38%), respectively (Fig. 5). The most drastic effect was achieved (from 0 to 73% soluble glucan) when the six single GTFB mutants were combined. Although water insoluble glucans mainly contain α-(1→3) linkages and soluble glucans mainly contain α-(1→6) glucosidic linkages (Shimamura *et al.*, 1994),

the linkage type and degree of branching of the glucans synthesized by these GTFB and GTFD site-directed mutants have not been reported; conceivably changes in either may have an effect on glucan solubility (Shimamura *et al.*, 1994).

Mutation W491G in conserved region III of GTFB of *S. mutans* GS5 resulted in complete loss of enzyme activity. This Trp residue is almost completely conserved in GTF enzymes (Tsumori *et al.*, 1997) (Fig. 5). Mutations in GTFA of *Lb. reuteri* 121, located near region IV, A1066N and H1065S:A1066S (making it more similar to GTFBIO) did not result in changes in glucosidic linkages synthesized. However, the double mutant showed a drastic change in enzyme activity: overall activity was lower than in wild type GTFA and no transferase activity could be measured (initial rates) (Kralj *et al.*, 2004c).

The GTF catalytic core usually starts with 2-4 conserved tyrosine residues (Fig. 5). Conversion of one to four of these tyrosine residues at position 169-172 in GTFB of *S. mutans* GS5 into alanine residues had little effect on overall activity. Only the adhesiveness of glucan products synthesized was altered by the mutations (Tsumori *et al.*, 1997).

An antibody directed against a 15 amino acid peptide (Fig. 5) extending from amino acid 368 to 382 in GTFC of *S. mutans* GS5 strongly inhibited GTF enzyme activity (Dertzbaugh & Macrina, 1990) (Fig. 5). Mutational analysis of the corresponding region, located at the N-terminal end of the catalytic domain, in GTFI of *S. downei* Mfe28 revealed that several residues in GTFI are important for activity. Mutations of Trp344Leu, Glu349Leu and His355Val resulted in a drop of activity of 30×, 4× and 7×, respectively (Monchois *et al.*, 1999c). Using chemical modification and monoclonal antibodies a region important for activity, corresponding to GTFC residues 435 to 453, distinct from the catalytic triad was identified (Funane *et al.*, 1993, Chia *et al.*, 1993). The one Asp and two Glu residues present were suggested to provide an essential carboxyl group (Funane *et al.*, 1993) (Fig. 5). The presence of functional carboxyl groups in this region has been confirmed by site-directed mutagenesis experiments (Fig. 5). Substitution of Asp511 and Asp513 of DSRS of *Ln. mesenteroides* NRRL-B512F in Asn resulted in complete loss and a strong decrease in glucan and oligosaccharide synthesizing activities, respectively (Monchois *et al.*, 1997). The similar residues in GTFB (Asp411 and Asp 413) and GTFC (Asp437 and Asp439) have been mutated to Asn residues. In GTFB these single mutations resulted in greatly reduced but detectable glucan synthesizing activity. However, simultaneously introduction of both mutations resulted in complete loss of enzyme activity. In contrast, these single amino acid mutations in GTFC, resulted in complete loss of enzyme activity (Chia *et al.*, 1998). Mutagenesis of GTFB Val412Ile and GTFC Val438Ile resulted in enhanced insoluble glucan synthesis of about 10 to 20%, whereas soluble glucan synthesis by these enzymes was significantly lower than for wild

type (Chia *et al.*, 1998). Mutagenesis of GTFB Glu422Gln and GTFC Glu448Gln resulted in 40% reduced glucan synthesizing activity.

PHYSIOLOGICAL FUNCTIONS OF GLUCANS OF LACTIC ACID BACTERIA

The physiological roles of glucans in lactic acid bacteria have not been clearly established, and are probably diverse and complex. The polymers may render protection to microbial cells in their natural environment, against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds (e.g. toxic metal ions, sulfur dioxide, ethanol, predation by protozoans, osmotic stress). Alternatively, they may also play a role in adhesion of cells to solid surfaces and biofilm formation, and also in cellular recognition (e.g. via binding to a lectin) (Cerning, 1990, Jolly *et al.*, 2002). Glucans may also contribute to increased oxygen tension and participate in the uptake of metal ions (Cerning, 1990).

Homopolysaccharides formed by oral streptoccocci apparently have a major influence on the formation of dental plaque. They are involved in adherence of bacteria to each other and to the tooth surface (Russell, 1990), modulating diffusion of substances through plaque and serving as extracellular energy reserves. Because of their clearly established role in dental caries formation (Balakrishnan *et al.*, 2000), most attention has been focussed on oral streptococci such as *S. mutans* and *Streptococcus salivarius* strains (Ebisu *et al.*, 1975, Shiroza & Kuramitsu, 1988, Rosell & Birkhed, 1974).

The presence of both glucan synthesizing and degrading enzyme(s) in a bacterium indicates that it can metabolize its polymer. Such observations have been made in streptococci (e.g. *S. mutans* and *Streptococcus sobrinus*), which hydrolyze the glucans (dextranases; Dex) produced by these plaque forming bacteria (Dewar & Walker, 1975, Igarashi *et al.*, 2002, Parker & Creamer, 1971, Colby *et al.*, 1995b, Colby *et al.*, 1995a).

APPLICATIONS OF POLY- AND OLIGOSACCHARIDES

EPS can be used as viscosifying, stabilizing, emulsifying, prebiotic, gelling or water binding agents in the food as well as in the non food industry (Sutherland, 1972). Dextran derivatives and activated dextrans are used in industrial and biomedical applications. (e.g, gel filtration products, blood plasma substitutes) (de Vuyst & Degeest, 1999).

Lactobacillus polysaccharides are of special interest because they may also contribute to human health as antitumoral (Oda *et al.*, 1983), antiulcer (de Roos & Katan, 2000), immunomodulating (Schiffrin *et al.*, 1995), or cholesterol-lowering (Roberfroid *et al.*, 1993) activity. Such beneficial properties of lactobacilli may be based on the polysaccharides and oligosaccharides produced.

Applications of glucans synthesized by lactobacilli

The GTF enzymes of *Streptococcus* sp. are generally produced constitutively (Kim & Robyt, 1994). GTF enzymes of *Leuconostoc* are specifically induced by sucrose, which is disadvantageous for several applications. Therefore, some constitutive *Ln. mesenteroides* mutants have been constructed (Kim & Robyt, 1994, Kitaoka & Robyt, 1998). Reuteransucrase from *Lb. reuteri* 121 is produced constitutively (van Geel-Schutten *et al.*, 1999). The GTF enzymes from *Lb. reuteri* 180 and *Lb. parabuchneri* 33 are also produced constitutively (unpublished data).

The only streptococcal species that is associated with food technology is *Streptococcus thermophilus*, which is used in the manufacture of yoghurt. The genus *Streptococcus* contains several well-known pathogens (e.g. *Streptococcus pneumoniae*) (Axelsson, 1998, Leroy & de Vuyst, 2004). Furthermore, glucans produced by oral streptococci play a key role in the cariogenesis process, by enhancing the attachment and colonization of cariogenic bacteria (Loesche, 1986). *Leuconostoc* strains play an important role in vegetable fermentations (Axelsson, 1998, Leroy & de Vuyst, 2004).

Lactobacilli are widespread in nature and many species have found applications in the food industry (e.g. in sourdough, *Lactobacillus sanfranciscensis*) (Axelsson, 1998, Leroy & de Vuyst, 2004). Several *Lb. reuteri* strains are able to produce anti-microbial metabolites (e.g. reutericyclin, reuterin and reutericin), which delay growth of some food borne pathogens (Kabuki *et al.*, 1997, Ganzle *et al.*, 2000, Talarico *et al.*, 1988). Furthermore, some *Lb. reuteri* strains have probiotic properties (see below) as has been demonstrated in various animals and humans (Casas *et al.*, 1998, Valeur *et al.*, 2004). The range of glucans and oligosaccharides produced by GTF enzymes present in lactobacilli (Kralj *et al.*, 2004d) may potentially act as prebiotics (see below) by stimulating the growth of probiotic strains or of beneficial endogenic strains of the gastrointestinal tract (Monsan & Paul, 1995, Olano-Martin *et al.*, 2000, Chung & Day, 2002).

Lactobacillus (*reuteri*) strains producing glucans thus possess the following general advantages: (i) constitutive GTF enzyme production, (ii) safe (GRAS status), and (iii) (potential) pro- and prebiotic properties (see below). Glucans from lactobacilli are therefore interesting and feasible alternatives for the additives currently used in the production of foods (e.g. sourdough, yoghurts, and health foods).

PROBIOTICS

A probiotic is a mono- or mixed culture of living microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous population of gastrointestinal microorganisms (Havenaar & Huis in 't Veld, 1992). Probiotic effects are considered to include inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, increase of the immune response and reduction of cholesterol levels (Du Toit *et al.*, 1998, Shornikova *et al.*, 1997).

Lactobacilli have been used as probiotics to control intestinal disorders such as lactose intolerance, acute gastroenteritis due to rotavirus and other enteric pathogens, adverse effects of pelvic radiotherapy, constipation, inflammatory bowel disease, and food allergy (Saavedra, 2001, Shornikova *et al.*, 1997, Salminen *et al.*, 1996). The beneficial effects of these organisms have been attributed to their ability to suppress the growth of pathogenic bacteria, possibly by secretion of antibacterial substances such as lactic acid, peroxide and bacteriocins (Salminen *et al.*, 1996). Their survival during the passage through the human gut, when administered in fermented milk products, has been investigated extensively in recent years. Well-controlled, small-scale studies on diarrhea in both adults and infants have shown that probiotics are beneficial and that they survive in sufficient numbers to affect gut microbial metabolism. Survival rates have been estimated at 20-40% for selected strains, the main obstacles for survival are gastric acidity and the action of bile salts. The carbohydrate-binding ability of a particular *Lb. reuteri* strain may be responsible for the adhesion to the mucosal surface of the intestine (Mukai *et al.*, 1998, Mukai *et al.*, 2004).

Lactobacillus reuteri ATCC 55730 was shown to colonize the human stomach, duodenum, and ileum. Furthermore, a clear immunomodulating effect in the human gut was observed (Valeur *et al.*, 2004). This probiotic strain produces a soluble glucan (reuteran), in which the majority of the linkages are of the α-(1→4) glucosidic type (~70%). This reuteran also contains α-(1→6) linked glucosyl units and 4,6-disubstituted α-glucosyl units at the branching points (Kralj *et al.*, 2004a). The preference for synthesizing α-(1→4) linkages is also evident from the oligosaccharides produced from sucrose with different acceptor substrates, e.g. isopanose from isomaltose. This *Lactobacillus* strain and its oligosaccharide and glucan products potentially is a synbiotic (combination of pro- and prebiotic, see below).

PREBIOTICS

Prebiotics are defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson & Roberfroid, 1995). Certain oligosaccharides and polysaccharides (iso-maltooligosaccharides, lactulose, fructans and oligosaccharides containing α-(1→2) glucosidic bonds) are resistant to digestion by enzymes of the gastro-intestinal tract (Bach Knudsen & Hessov, 1995, Rumessen & Gudmand-Hoyer, 1998, Cummings *et al.*, 2001, Buchholz & Monsan, 2003). Fructooligosaccharides are resistant to digestion because of the presence of fructose units in the β-form. These oligosaccharides can, however, be fermented by beneficial microorganisms**,** such as bifidobacteria (Van Laere *et al.*, 2000). Prebiotics therefore, selectively stimulate the growth of these microorganisms (Casas *et al.*, 1998, Salminen *et al.*, 1996). For inulin it

was shown that when orally applied it reduces colitis (inflammatory bowel disease) in rat intestine (Videla *et al.*, 2001).

Examples of the stimulating effect of pre- and synbiotics are: (i) addition of fructooligosaccharides causing an increase in the number of bifidobacteria *in vitro* (Gmeiner *et al.*, 2000), (ii) upon administration of honey (honey contains in decreasing order fructose, glucose, maltose, and sucrose) to rats the number of lactic acid bacteria in the intestine increased dramatically (Shamala *et al.*, 2000), (iii) mixtures of *Lactobacillus acidophilus* 74-2 and fructooligosaccharides gave rise to an increase in bifidobacteria in an artificial human intestinal microbial ecosystem (Gmeiner *et al.*, 2000), (iv) a prebiotic effect of α-glucooligosaccharides has been demonstrated for piglets, broilers and calves: addition of 0.15% (w/w) of α-glucooligosaccharides to young calves' feed for instance resulted in a 20% decrease of the veterinary costs (Monsan & Paul, 1995), (v) another study showed that α-glucooligosaccharides containing α-(1→2) glucosidic bonds promote the growth of the cellulolytic intestinal flora (Djouzi & Andrieux, 1997), and (vi) they also induced a broader range of glycolytic enzymes than fructooligosaccharides and galactooligosaccharides, without any important side-production of gasses and thus no detrimental effects (Djouzi & Andrieux, 1997). In short, gluco-, fructooligosaccharides, and inulin are of increasing interest to clinical nutritionists as functional food additives.

CONCLUSIONS

Glucansucrases are composed of four different domains, with a highly conserved catalytic core containing a permuted $(\beta/\alpha)_8$ barrel structure. The different glucansucrase enzymes are highly similar in their catalytic core. Nevertheless, they synthesize various α-glucosidic bonds (e.g. [α-(1→2)], [α-(1→3)], [α-(1→4)], and [α-(1→6)] in their polymer and oligosaccharide products resulting in a large variation of properties. Since there is no detailed structural information, the basis of linkage specificity is poorly understood.

SCOPE OF THIS THESIS

This thesis focuses on structure/function relationships of glucansucrase enzymes of lactobacilli. The first glucansucrase charcaterized from a *Lactobacillus*, GTFA of *Lb. reuteri* 121 synthesizes a unique glucan polymer containing large amounts of α-(1→4) linkages. All other glucansucrases known (from streptococci and leuconostocs) synthesized glucans with different ratios of α-(1→3) and α-(1→6) linkages. The aims of the research described in this thesis were (i) to analyze a possibly wider distribution of glucansucrase genes and enzymes in lactobacilli, (ii) to isolate and characterize the glucansucrase genes and enzymes from different lactobacilli and analysis of their oligosaccharides and glucan products (iii) to identify GTF regions important in glucan structure determination (by applying site-directed mutagenesis) (iv) to obtain a better

understanding of GTF features determining linkage specificity within the oligo- and polysaccharide products synthesized.

Chapter 1 reviews current knowledge about the synthesis of glucans by glucansucrase enzymes from lactic acid bacteria and their applications.

In **Chapter 2** a molecular approach was chosen to clone the gene encoding the glucansucrase (reuteransucrase) from *Lb. reuteri* 121. Conclusive evidence is presented that the heterologously produced glucansucrase is responsible for reuteran production by *Lb. reuteri* 121.

In **Chapter 3** a biochemical and molecular characterization of the *Lb. reuteri* 121 reuteransucrase (GTFA) is presented, including deletion analysis of its glucan binding domain, identification of its catalytic residues and characterization of oligosaccharides synthesized from sucrose with various acceptor substrates.

In **Chapter 4** the isolation and subsequent molecular and biochemical characterization of a second reuteransucrase, isolated from a probiotic *Lb. reuteri* "BioGaia" strain is described. This enzyme synthesizes the largest amount of α-(1→4) linkages in its glucan (up to 70%) and oligosaccharide products reported to date. Furthermore, properties of the two reuteransucrase enzymes currently known are compared.

Chapter 5 describes identification of regions in GTFA of *Lb. reuteri* 121 which are important for α-(1→4) glucosidic bond specificity. A triple amino acid mutation resulted in a drastic decrease in α-(1→4) glucosidic bonds formed. The combination of this mutant with a double mutant located in a different region resulted in an even further decrease of α-(1→4) glucosidic bonds synthesized. Also within oligosaccharides synthesized by the different mutants the same shifts in linkages synthesized could be observed.

Chapter 6 shows that the genus *Lactobacillus* contains a wide variety of (putative) glucansucrase gene fragments.

In **Chapter 7** glucansucrase genes from different lactobacilli are isolated, cloned and expressed in *E. coli*, showing that also the genus *Lactobacillus* synthesizes a wide variety of glucansucrase genes, enzymes and glucan products (dextran, mutan) as is observed for members of the genera *Streptococcus* and *Leuconostoc*.

Chapter 2

Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucan with α-(1→4) and α-(1→6) glucosidic bonds

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SUMMARY

Lactobacillus reuteri strain 121 produces a unique, highly branched, soluble glucan in which the majority of the linkages are of the α-(1→4) glucosidic type. The glucan also contains α-(1→6) linked glucosyl units and 4,6-disubstituted α-glucosyl units at the branching points. Using degenerate primers, based on the amino acid sequences of conserved regions from known glucosyltransferase (*gtf*) genes from lactic acid bacteria, the *Lb. reuteri* strain 121 glucosyltransferase gene (*gtfA*) was isolated. The size of the *gtfA* ORF was 5343 bp, encoding a protein of 1781 amino acids with a deduced M_r of 198,637 Da. The deduced amino acid sequence of GTFA revealed clear similarities with other glucosyltransferases. GTFA has a relatively large variable N-terminal domain (702 amino acids) with five unique repeats, and a relatively short C-terminal domain (267 amino acids). The *gtfA* gene was expressed in *Escherichia coli*, yielding an active GTFA enzyme. With respect to binding type and size distribution, the recombinant GTFA enzyme and the *Lb. reuteri* strain 121 culture supernatants synthesized identical glucan polymers. Furthermore, the deduced amino acid sequence of the *gtfA* ORF and the N-terminal amino acid sequence of the glucosyltransferase isolated from culture supernatants of *Lb. reuteri* strain 121 were the same. GTFA is thus responsible for the synthesis of the unique glucan polymer in *Lb. reuteri* strain 121. This is the first report on the molecular characterization of a glucosyltransferase from a *Lactobacillus* strain.

INTRODUCTION

Many lactic acid bacteria employ large extracellular enzymes, glucosyltransferases (EC 2.4.1.5, commonly named glucansucrases, GTFs), for the synthesis of high molecular weight α-glucans from sucrose. Moreover, low molecular weight oligosaccharides are produced in the presence of suitable acceptor molecules.

The GTFenzymes of oral streptococci and the dextran- and alternansucrases from *Leuconostoc mesenteroides* strains have been studied in most detail. All GTFs from lactic acid bacteria share a common structure and are composed of four distinct domains: their N-terminal end starts with (i) a signal peptide of 32-34 amino acids, followed by (ii) a highly variable stretch of 123-129 amino acids, (iii) a highly conserved catalytic or sucrose binding domain of about 1,000 amino acids and (iv) a C-terminal glucan binding domain of about 500 amino acids, composed of a series of tandem repeats (Monchois *et al.*, 1999d).

Amino acid sequence comparisons revealed that GTFs possess a $(\beta/\alpha)_8$ barrel structure similar to glycoside hydrolases of family 13. This family includes for instance α-amylase and cyclodextrin glycosyltransferase (CGTase) (van der Veen *et al.*, 2000).

The core of the proteins belonging to this family is constituted of 8 β-sheets alternated with 8 α-helices. In GTFs, however, this $(\beta/\alpha)_8$ barrel structure is circularly permuted (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997). Therefore GTFs are classified in family 70 of glycoside hydrolases (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Some GTF enzymes, such as dextransucrase from *Ln. mesenteroides* strain B-512F, catalyze the formation of linear glucans containing mostly α-(1→6) linkages (dextrans). Other types synthesize dextrans with α-(1→2), α-(1→3) or α-(1→4) branches (Monchois *et al.*, 1999d, Funane *et al.*, 2001). Alternansucrase from *Ln. mesenteroides* strain NRRL B-1355 synthesizes a glucan with alternating α-(1→6), and α-(1→3) glucosidic bonds (Arguello-Morales *et al.*, 2000). Recently, a GTF of *Ln. mesenteroides* strain NRRL B-1355 has been characterized, synthesizing a glucan containing α-(1→2) glucosidic linkages (Smith *et al.*, 1998). There are few reports, however, about glucan synthesis in lactobacilli (van Geel-Schutten *et al.*, 1998). A biochemical and molecular characterization of the enzyme(s) responsible for glucan synthesis in lactobacilli has not been reported.

In previous studies we have isolated a strain of *Lactobacillus reuteri*, capable of producing both a fructan and a glucan. Depending on the carbon source in the culture medium, the GTF responsible for the synthesis of this polysaccharide material is completely cell-associated or partly released into the culture medium (van Geel-Schutten *et al.*, 1998, van Geel-Schutten *et al.*, 1999). This paper describes the first molecular characterization of a *Lactobacillus* glucosyltransferase gene (*gtfA*), expression of *gtfA* in *Escherichia coli*, and the characterization of its glucan product. The *gtfA* gene encodes a novel type of glucosyltransferase, synthesizing a highly branched glucan with a unique structure containing α-(1→4) and α-(1→6) linkages.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions

Lactobacillus reuteri strains 121 and 35-5 (both obtained from the culture collection of TNO Nutrition and Food Research, Zeist, The Netherlands) were cultivated anaerobically at 37 °C in MRS medium (Difco) (De Man *et al.*, 1960) or in MRS-s medium (i.e. MRS-medium with 100 g l^{-1} sucrose instead of 20 g l^{-1} glucose). *Escherichia coli* DH5α (Phabagen) (Hanahan, 1983), *E. coli* JM 109 (Promega) (Hanahan, 1983), and *E. coli* TOP 10 (Invitrogen) were used as hosts for cloning purposes. Plasmids pCR2.1-TOPO (Invitrogen), pCR-XL-TOPO (Invitrogen), and pEMBL8 (Dente *et al.*, 1983) were used for cloning of the glucosyltransferase gene for sequencing purposes. Plasmid pBluescript II SK^+ (Stratagene) was used for cloning of the complete glucosyltransferase gene. Plasmid pET15b (Novagen) was used for expression of the *gtf* gene in *E. coli* BL21

Star (DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium (Ausubel *et al.*, 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (100 μg ml^{-1} ampicilline or 50 μg ml^{-1} kanamycine). Agar plates were made by adding 1.5% agar to the LB medium; when appropriate X-gal (20 μg ml^{-1}) was added.

Isolation of DNA

Lb. reuteri strain 121 total DNA was isolated according to (Nagy *et al.*, 1995). Plasmid DNA of *Lb. reuteri* strain 121 was isolated using a modification of the methods described (Anderson & McKay, 1983, Burger & Dicks, 1994). Prewarmed (37 °C) fresh MRS broth (10 ml) was inoculated with 200 μl of an overnight culture and incubated for 2.5 h at 37 °C. Cells were harvested by centrifugation and washed with 2 ml sterile STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8). After centrifugation, the pellet was resuspended in 380 μl solution I (0.5 M sucrose, 50 mM Tris/HCl, 1 mM EDTA, pH 8, containing 2 mg/ml lysozyme (Sigma) and 6.6 U mutanolysin (Sigma). After 1.5 h of incubation at 37 °C, 50 μl of solution II (0.25 M EDTA, 50 mM Tris-HCl, pH 8) and 30 μl of solution III (20% SDS, 50 mM Tris-HCl, 20 mM EDTA, pH 8) were added and the suspension was mixed. NaOH (30 μl of 3 M solution) was added, followed by 50 μl 2 M Tris/HCl and 72 μl 5 N NaCl. After extraction with equal volumes of phenol and chloroform, the DNA was precipitated with ethanol as described (Sambrook *et al.*, 1989).

Plasmid DNA of *E. coli* was isolated using the alkaline lysis method (Birnboim & Doly, 1979) or with a Wizard Plus SV plasmid extraction kit (Promega).

Molecular techniques

General procedures for cloning, *E. coli* transformations, DNA manipulations, and agarose gel electrophoresis were as described (Sambrook *et al.*, 1989). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the enzyme suppliers (New England Biolabs; Roche Biochemicals). Primers were obtained from Eurogentec. Sequencing was performed according to (Sanger *et al.*, 1977). DNA was amplified by PCR on a DNA Thermal Cycler 480 (Perkin Elmer, Boston, MA) using *ampliTAQ*-DNA polymerase (Perkin Elmer) or *Pwo* DNA polymerase (Roche Biochemicals). For inverse PCR the Expand High Fidelity PCR system (Roche Biochemicals) was used as described by the supplier. Fragments were isolated from agarose gels using a Qiagen gel extraction kit (Qiagen) following the instructions of the supplier.

For Southern hybridization, DNA was restricted with endonucleases, separated by agarose gel electrophoresis, and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech) following the manufacturer's instructions. The probes, which were

labeled with digoxigenin-dUTP, were prepared and patterns of hybridization with the probes were examined with a digoxigenin DNA Labeling and Detection kit (Roche Biochemicals), following the manufacturer's instructions. Stringent probe hybridizations were performed at 65 °C and non-stringent hybridizations were performed at 45 °C.

Identification and nucleotide sequence analysis of the glucosyltransferase gene

An overview of the isolation strategy of the *gtf* gene is given in Fig. 1. The first

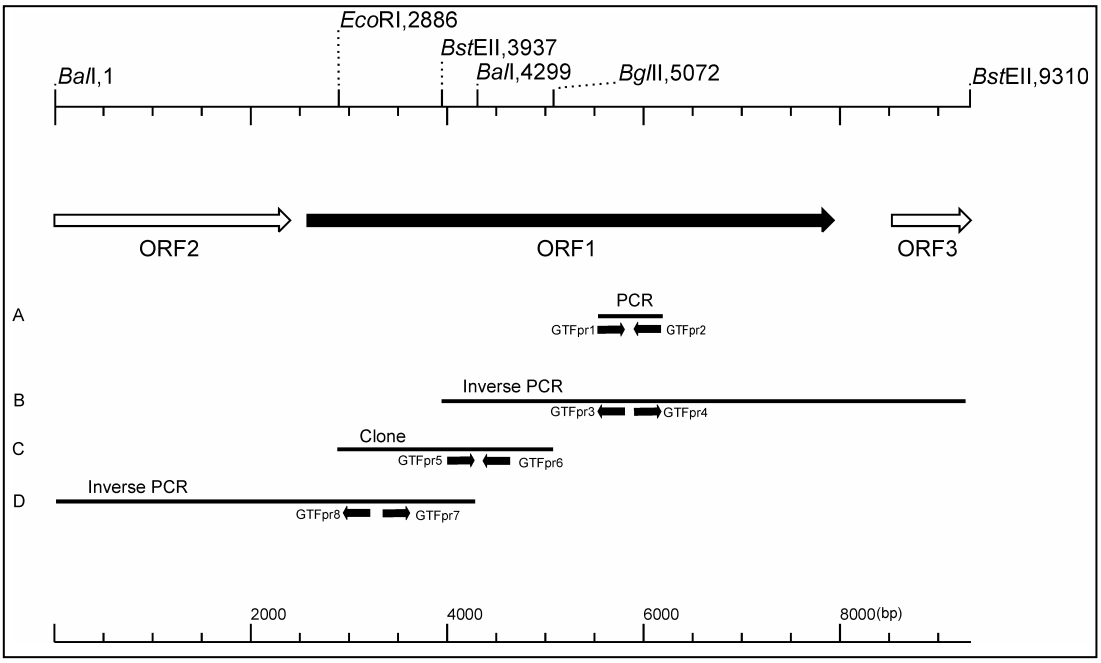

Figure 1. Strategy used for the isolation of the *gtfA* gene and surrounding regions from *Lb. reuteri* strain 121 chromosomal DNA. Primers are indicated with small arrows. The fragment given in Fig. 1A is the 659 bp insert isolated with degenerate primers. The fragments in Fig. 1B (5229 bp) and 1D (4229 bp) are regions amplified by inverse PCR. The fragment in Fig. 1C (2186 bp) was obtained by colony blot hybridization of a partial gene library.

part of the glucosyltransferase gene was isolated by amplification of chromosomal DNA of *Lb. reuteri* strain 121 with PCR using degenerate primers (GTFpr1 5'-GAYAAKWSNAAKSYNRTNGTNSARGC-3' and GTFpr2 5'-ANRTCNCCRTARTA NACNCKNG-3' ; Y=T or C, K=G or T, W= A or T, S = C or G, R = A or G , N = A, C, G or T) based on conserved amino acid sequences, present in the catalytic core, deduced from the glucosyltransferase genes of *Streptococcus downei (gtfS)*, *Streptococcus mutans (gtfC)*, *S. downei* (*gtfI*), *Streptococcus salivarius* (*gtfK* and *gtfM)*, and *dsrA* of *Ln. mesenteroides* (Gilmore *et al.*, 1990, Ueda *et al.*, 1988, Ferretti *et al.*, 1987, Giffard *et al.*, 1993, Simpson *et al.*, 1995b, Monchois *et al.*, 1996), An amplification product with the expected size of about 660 bp was obtained and cloned in *E. coli* JM 109 using the

pCR2.1 vector. Analysis of its nucleotide sequence (659 bp, Fig. 1A) confirmed its glucosyltransferase identity. The 659 bp amplified fragment was used to design the primers GTFpr3 5'-GGGCTTTCAGATGCAACTAATCGTTGGGG-3' and GTFpr4 5'-GCTTATTTGAGACAGCATCTGAGTCCATACC-3' for inverse PCR. *Lb. reuteri* strain 121 chromosomal DNA was digested with *Bst*EII and ligated, yielding circular DNA molecules. PCR with diverging primers GTFpr3 and GTFpr4 with the circular ligation products as template yielded an amplicon of 5229 bp (Fig. 1B), that was cloned into pCR-XL-TOPO (Invitrogen). Based on its nucleotide sequence, a probe was designed encompassing GTFpr5 5'-GGTAATGATAAGCGCCATACAG-3' and GTFpr6 5'-GATATACTGCTGCTACATCTGGAC-3' to screen a partial gene library of *Lb. reuteri* strain 121. This library was constructed by ligating *Eco*RI/*Bgl*II fragments of genomic DNA into the *Eco*RI/*Bam*HI sites of pEMBL8. A positive clone was selected by colony blot hybridization (Datta & MacQuillan, 1987) using hybond N-filters (Amersham Pharmacia Biotech), and its nucleotide sequence was determined (2186 bp, Fig. 1C). This information was used to design the diverging primers GTFpr7 5'-CGGGATTCAATTGAAATTGTTAGTCGATACAG-3' and GTFpr8 5'-GAGATGCGT TCGTTGCATTCCATCCTGAAAC-3' for an inverse PCR step with *Bal*I digested, and ligated, chromosomal DNA. The inverse PCR fragment obtained was cloned into pCR-XL-TOPO (Invitrogen) and sequenced (4299 bp, Fig. 1D).

Construction of plasmids for expression of the glucosyltransferase gene in *E. coli*

Two separate PCR reactions with primers GTFpr11-14 were used to amplify the complete *gtf* gene (Fig. 2). The product of the first PCR reaction, synthesized with primers GTFpr11 5' GATGCAT**GAGCTC**CCATGGACCAACAAGTTCAGCAAGCTT CC-3', containing *Sac*I (bold) and *Nco*I (underlined) sites, and GTFpr12 5'- GTGCATTA AAGTACGTAACCAATCAGTATTTCCGG-3', was digested with *Sac*I/*Pst*I. The product of the second PCR reaction, primed with oligo's GTFpr13 5'-TTGATGGTATGGTGGCCTAATACTCTTACCC and GTFpr14 5'- ATATCGAT**GGG CCC**CGGATCCTATTA*GTGATGGTGATGGTGATG*TAGTTTATTTTGATCAAGCAT CTTACC-3', containing *Apa*I (bold), *Bam*HI (underlined) and a 6x His-tag (italics) was digested with *Pst*I/*Apa*I. The resulting fragments (2547 bp) and (2723 bp) were cloned in the corresponding sites of pBluescript II SK^+, yielding pBSP2500 and pBPA2700. Both plasmids were digested with *Apa*I/*Pst*I and the fragment from pBPA2700 containing the 3' part of *gtf* was ligated into pBSP2500 yielding pBGTF1. Plasmid pBGTF1 was digested with *Nco*I/*Bam*HI and the resulting 5.3 kb fragment was ligated into the corresponding sites of the expression vector pET15b (Novagen) yielding p15gtf.

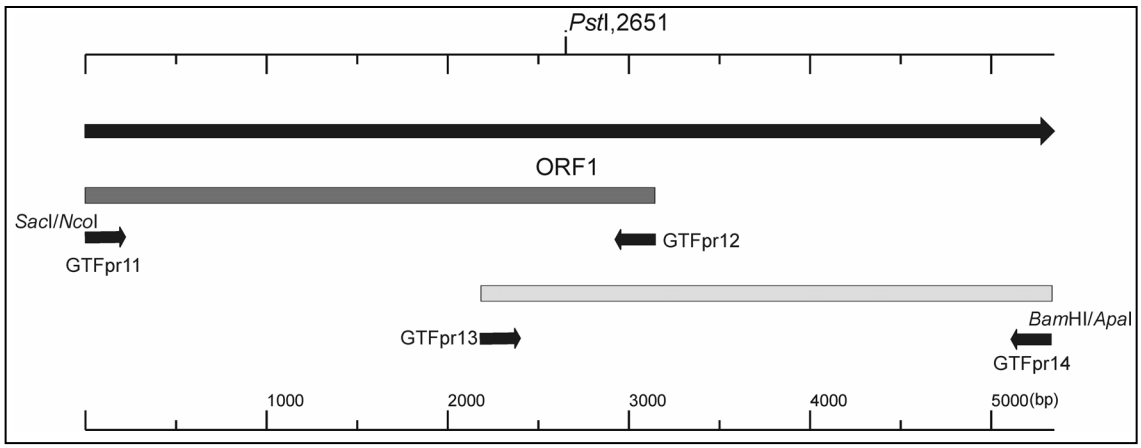

Figure 2. Overview of the primers and restriction sites used for cloning of the *gtfA* gene in the expression vector pET15B (Novagen).

Dendrogram construction

Amino acid sequences were aligned with Clustal W 1.74 (Higgins & Sharp, 1988) with a gap opening penalty of 30 and a gap extension penalty of 0.5. Amino acid sequences were obtained from GenBank and divided into the following three groups: (1) DSRB of *Ln. mesenteroides* NRRL B-1299 (AAB95453), DSRS of *Ln. mesenteroides* NRRL B-512F (AAA53749), DSRA of *Ln. mesenteroides* NRRL B-1299 (AAB40875), ASR of *Ln. mesenteroides* NRRL B-1355 (CAB65910), DSRT of *Ln. mesenteroides* NRRL B-512F (BAA90527) and, GTFA of *Lb. reuteri* strain 121; (2) GTFIa of *Streptococcus sobrinus* OMZ176 (BAA14241), GTFI of *S. downei* Mfe28 (BAA0296), GTFIs of *S. sobrinus* OMZ176 (BAA02976), GTFB of *S. mutans* GS5 (AAA88588) and, GTFC of *S. mutans* GS5 (AAA88589); (3) GTFG of *Streptococcus gordonii* (AAC 43483), GTFR of *Streptococcus oralis* ATCC 10557 (BAA95201), GTFS of *S. downei* Mfe28 (AAA26898), GTFM of *S. salivarius* ATCC 25975 (AAC41413), GTFL of *S. salivarius* ATCC 25975 (AAC41412), GTFN of *S. salivarius* ATCC 25975 (AAC05165), GTFD of *S. mutans* GS5 (AAA26895), GTFJ of *S. salivarius* ATCC 25975 (CAA77900), GTFT of *S. sobrinus* OMZ176 (D13928) and, GTFK of *S. salivarius* ATCC 25975 (CAA77898). Amino acid sequences were aligned first within each group. The complete alignment was performed by aligning groups 1 to 3 to each other. Tree construction was performed using TreeCon 1.3b (no correction for distance estimation, 10 bootstrap samples, using the neighbor joining algorithm) (van de Peer & De Wachter, 1994).

Preparation of *E. coli* cell free extracts

Cells of *E. coli* BL21star (DE3) harboring p15gtf were harvested by centrifugation (10 min at 4 °C at 10,000 × *g*) after 16 h of growth. The pellet was washed with 50 mM sodium acetate buffer pH 5.5 containing 1 mM $CaCl_2$ and 1% (v/v) Tween 80 and the suspension was centrifuged again (10 min at 4 °C at 10,000 × *g*). Pelleted cells were

resuspended in 50 mM sodium acetate buffer pH 5.5 containing 1 mM $CaCl_2$, 1% (v/v) Tween 80, and 5 mM β-mercaptoethanol. Cells were broken by sonication (7 × 15 sec at 7 micron with 30 sec intervals). Cell debris and intact cells were removed by centrifugation for 20 min at 4 °C at 10,000 × *g* and the resulting cell free extract (supernatant) was used in the enzyme assays.

Enzyme assays

Using *E. coli* cell free extracts or *Lb. reuteri* strain 121 grown on MRSs culture supernatant as source of enzyme, glucosyltransferase activity was measured by determining the release of fructose from sucrose at 37 °C in 50 mM sodium acetate buffer pH 5.5 containing 1 mM $CaCl_2$ and 100 mM sucrose (van Geel-Schutten *et al.*, 1999).

Amino acid sequence determination from the glucosyltransferase from *Lb. reuteri* strain 121 using two-dimensional polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE, i.e. iso-electricfocussing followed by SDS-PAGE) experiments were performed as described earlier (O'Farrell, 1975), using the II xi Cell system according to the manufacturer's instructions (Bio-Rad). Chemicals and materials were from Bio-Rad, unless indicated otherwise.

(i) Sample preparation. Supernatants of cultures of *Lb. reuteri* strain 121 grown on MRSs (10 ml precipitated with 2 ml 30% TCA) in the exponentional phase of growth, harvested by centrifugation (10,000 × *g* at 4 °C for 10 min), were used as source of glucosyltransferase protein.

(ii) Glucosyltransferase activity staining. After separation in the second dimension, glucosyltransferase activity was identified as described (van Geel-Schutten *et al.*, 1999), using a PAS staining. The same gel was silver stained (Genotech) to identify other proteins on the gel, thereby facilitating the identification of the glucosyltransferase spot on the blot (see below).

(iii) Amino acid sequencing. A second gel obtained after 2D-PAGE, identical to the glucosyltransferase activity stained gel, was blotted according to (Kyhse-Andersen, 1984) to a PVDF membrane (Roche Biochemicals) with a "semi-dry electroblotter" (Ancos). The PVDF membrane was stained with Coomassie Brilliant Blue R-250. The N-terminal amino acid sequence of the protein band which corresponded with the activity band obtained by PAS staining was determined on a Perkin Elmer ABI 476A automated sequencer using Edman degradation (NAPS Protein Sequencing and Peptide Mapping Laboratory).

Characterization of the glucans produced

Glucans were produced by incubating the enzyme preparations overnight, using the conditions described above under enzyme assays. Glucans produced by *Lb. reuteri* strain 121, strain 35-5 (producing only the wild type glucan and not the fructan), and glucans produced with the glucosyltransferase expressed in *E. coli*, were isolated by precipitation with ethanol (van Geel-Schutten *et al.*, 1999). NMR spectroscopy and methylation analysis were performed as described earlier (van Geel-Schutten *et al.*, 1999). The molecular weights of the glucans were determined by high performance size exclusion chromatography (HPSEC) coupled on-line with a multi angle laser light scattering (MALLS) and differential refractive index detection (RI, Schambeck SDF). The HPSEC system consisted of an isocratic pump, an injection valve, a guard column and a set of two SEC columns in series (Shodex SB806MHQ column and TSK gel 6000PW). A Dawn-DSP-F (Wyatt Technology) laser photometer HeNe (λ = 632.8 nm) equipped with a K5 flow cell, thermostatted by a Peltier heating system, was used as MALLS detector. Samples were filtered through a 0.45 µm filter (MILLEX) and the injection volume was 220 µl. Na_2SO_4 (0.1 M) was used as eluent at a flow rate of 0.8 ml min^{-1}. Pullulan and dextran samples with Mw ranging from 4.10^4 to 2.10^6 Da were used as standards. Determinations were performed in duplicate.

Nucleotide accession number

The nucleotide sequence of *gtfA* has been assigned accession no. AX306822 by GenBank.

RESULTS

Isolation and nucleotide sequence analysis of the putative *Lb. reuteri* strain 121 glucosyltransferase gene

Based on sequence homology between conserved regions, located in the catalytic core of different *gtf* genes of Gram-positive bacteria, degenerate primers were designed and used for PCR with chromosomal DNA of *Lb. reuteri* strain 121 as template. A single fragment of 659 bp (Fig. 1) was obtained and sequence analysis confirmed its *gtf* identity. Southern hybridization of chromosomal DNA of *Lb. reuteri* strain 121 with this 659 bp amplified PCR fragment, followed by washing under non-stringent conditions revealed one hybridizing fragment. Plasmid DNA of *Lb. reuteri* did not show hybridization.

In subsequent steps a total of 9310 bp was obtained and sequenced (Fig. 1). One complete ORF and two partial ORFs were located on this compiled sequence: ORF1 (5343 bp, Fig. 1), encoding a putative glucosyltransferase (GTFA), ORF2 (2403 bp) upstream of ORF1, and ORF3 (786 bp), downstream of *gtfA*. The deduced amino acid

sequence of ORF2 showed homology with glucosyltransferases, whereas the deduced amino acid sequence of ORF3 did not show significant homology to any protein present in databases. The g*tfA* gene encodes a putative protein of 1781 amino acids, with a deduced molecular weight of 198,637 Da and a pI of 5.04. It was preceded by a putative ribosomal binding site (GAAGGAG), localized 6 bp upstream from the ATG-start codon. According to the consensus promoter sequences described previously for lactobacilli (Pouwels & Leer, 1993), a potential promoter sequence in the upstream sequence of *gtfA* (42 bp from the start codon) could be identified with a -35 sequence (TTGAAA) separated by 19 bp from a -10 sequence (TATAAT).

Two inverted repeats were located 61 bp downstream from the *gtfA* termination codon. These repeats could form a stem (20 bp) loop (12 nucleotides) secondary structure with a ΔG value of –20.4 kcal mol^{-1}, followed by a series of thymidin residues, suggesting a Rho independent transcription termination signal (Bektesh & Richardson, 1980).

Amino acid sequence alignments of *Lb. reuteri* GTFA with other glucosyltransferases

Alignment of the amino acid sequence of GTFA with other glucosyltransferases using Blast (Altschul *et al.*, 1990), revealed clear similarities. Highest similarity (46% identity, 59% similarity within 1261 amino acids) at the amino acid level was found with alternansucrase from *Ln. mesenteroides* NRRL B-1355. The putative protein structure of GTFA was similar to that of other glucosyltransferases containing (i) a N-terminal signal sequence of 38 amino acids, (ii) a relatively large variable N-terminal domain of 702 amino acids (iii) a catalytic domain of 774 amino acids, and a C-terminal domain of 267 amino acids (Fig. 3).

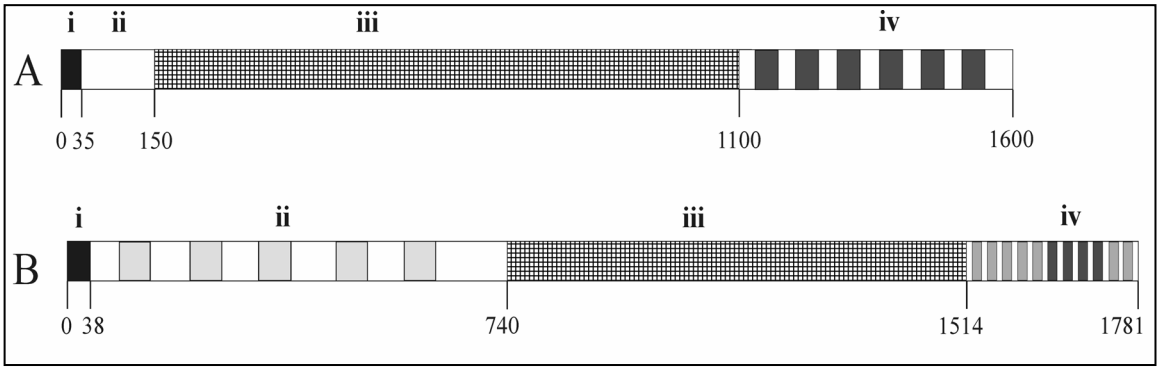

Figure 3. Schematic representation of **A**) glucosyltransferases in general and **B**) GTFA of *Lb. reuteri* strain 121, showing the four different domains i) N-terminal signal sequence; ii) variable region with 5 RDV repeats (light grey boxes); iii) catalytic domain; iv) C-terminal (putative) glucan binding domain with 4 YG-repeating units (dark grey boxes) according to the definition of (Giffard & Jacques, 1994) and 7 less conserved YG-repeating units (light grey boxes).

```
RDV1      174--RPDVKNVH-NVYNADNSGFDVNVNIDFSKMKDYRDSIEIVSRYS--224
RDV2      290--RPDVAKVYPQVVGAANSGFNVTFNI--SDL-DYTHQYQVLSRYS--338
RDV3      411--RPDVAKAYPTVKTATNSGFKVTFKV--NNL-QPGHQYSVVSRFS--459
RDV4      528--RPDVAAVYPSLYNSAVSGFDTTIKLTNDQYQALNGQLQVLLRFS--579
RDV5      654--RNDVYKVNPAIENSSMSGFQGIITLP---VTVKNENVQLVHRFS--702
               * **  .   :  :  ***.  ..:          . . ::*:*
```

Figure 4. Alignment of the five unique repeat elements (RDV1-RDV5) from the N-terminal region of GTFA of *Lb. reuteri* strain 121. The conserved (**.**), highly conserved (**:**) and identical (*****) residues are indicated. The RDV motif is indicated in bold type R(N/P)DV. Two other conserved motifs, SGF and R(F/Y)S, are indicated by boxes.

The deduced N-terminal amino acid sequence of GTFA contained a putative secretion peptide with a predicted signal peptidase cleavage site (SPase) between amino acid 38 and 39 (http://www.cbs.dtu.dk/services/SignalP/). To confirm this cleavage site, the *Lb. reuteri* strain 121 enzyme was purified by 2D-PAGE and subjected to N-terminal sequence analysis. The first 13 amino acids were identified as: DQQVQQASTLQDQ; except for the tenth residue the sequence was identical to that of the deduced amino acid sequence following the predicted cleavage site. 2D-PAGE experiments also confirmed the predicted M_r and pI of GTFA. Within the deduced N-terminal variable region of GTFA, a series of five repeating units were found. These repeating units, designated RDV, were on average 41 amino acids long, seperated on average by 71 amino acids (Fig. 4). These repeats have never been seen in other GTFenzymes and showed no significant homology to any protein motifs present in databases.

```
              ◊ ⇓  ∇∇                                          ⇓∇∇∇
ASR      633-RVDAVDNVDADLLKIAGDYFKALYGTDKSDANANKHLSILEDWNG-677
GTFD     463-RVDAVDNVNADLLQIASDYLKAHYGVDKSEKNAINHLSILEAWSD-507
DSRS     549-RVDAVDNVDADLLQIAADYFKLAYGVDQNDATANQHLSILEDWSH-593
GTFA    1022-RVDAPDNIDADLMNIAQDYFNAAYGMD-SDAVSNKHINILEDWNH-1065
             **** **:***::** **::  ** * .:  : :*:.*** *.

AS       292-RMDAVAFIWKQMQT--305              333-FKSEAIVH-340
```

Figure 5. Alignment of parts of the catalytic cores of DSRS of *Ln. mesenteroides* NRRL B-512F (Wilke-Douglas *et al.*, 1989), GTFD of *S. mutans* GS5 (Fujiwara *et al.*, 1998), ASR of *Ln. mesenteroides* NRRL B-1355 (Arguello-Morales *et al.*, 2000), GTFA of *Lb. reuteri* strain 121 and amylosucrase (AS) of *N. polysaccharea* (De Montalk *et al.*, 1999). ***** , identical residue; **:** , highly conserved residue; **.** , conserved residue; ⇓, putative catalytic residue; ∇, residue possibly playing a role in binding of acceptor molecules and in the transfer of the glucosyl residue; ◊, putative chloride binding site; **AA** amino acids which are conserved in all glucosyltransferases, but not in GTFA. Adapted from (Monchois *et al.*, 1999d).

The putative catalytic domain of GTFA showed high similarity (about 45% identity and 60% similarity) to other known streptococcal and *Leuconostoc* GTF enzymes. However, not all of the conserved amino acids found in the other glucosyltransferases

were found in the deduced amino acid sequence of GTFA. Particularly in the region downstream of Asp[1024], 3 out of 10 conserved amino acids were not found in GTFA (Fig. 5). One of the conserved amino acid substitutions in this region of GTFA, Ile[1029], was also found in amylosucrase, a glucosyltransferase from *Neisseria polysaccharea* synthesizing an α-(1→4) glucan (De Montalk *et al.*, 1999).

The relatively short C-terminal domain of GTFA contains four YG-repeating units according to the definition of (Giffard & Jacques, 1994) and seven YG-repeating units which are less conserved (Fig. 6).

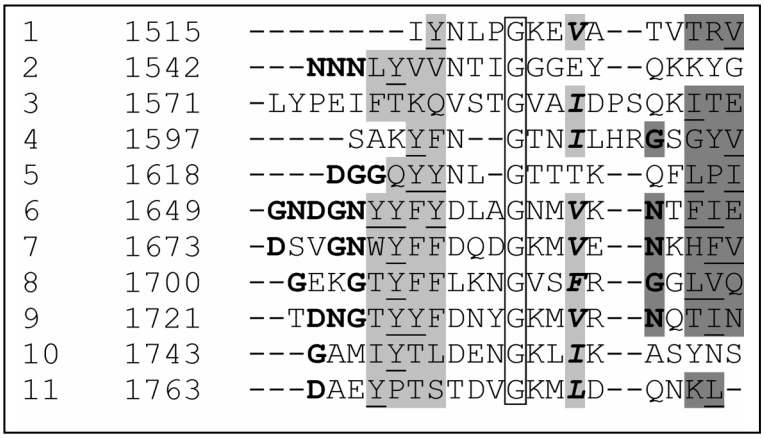

Figure 6. Alignment of the repeats in the C-terminal region of GTFA of *Lb. reuteri* strain 121. YG-repeats consist of, from left to right: (i) 4-6 residues that usually include glycine and/or aspartate and/or asparagine residues (indicated in bold type), (ii) a cluster of 1-4 residues (lightly shaded) that nearly always includes tyrosine (underlined), (iii) 3-4 poorly conserved residues (iv) a glycine residue (boxed), (v) 2 poorly conserved residues, (vi) a residue that is usually hydrophobic (indicated in bold type italics and lightly shaded), (vii) 1 poorly conserved residue, (viii) a neutral polar residue (darkly shaded) that is usually glycine or asparagine (indicated in bold type). (ix) 1 poorly conserved residue and, (x) 3 residues (darkly shaded) that include 1-3 hydrophobic residues (underlined) (Giffard & Jacques, 1994). Four repeats (6, 7, 8 and 9) match exactly the consensus sequence defined by Giffard (Giffard & Jacques, 1994). The other seven show lower homology with the YG-repeats.

Dendrogram

Construction of a dendrogram (Fig. 7), based on the complete amino acid sequences of different glucosyltransferases of lactic acid bacteria, revealed that GTFA of *Lb. reuteri* strain 121 is distinct from other glucosyltransferases known. Again, GTFA is most closely related to the alternansucrase (ASR) of *Ln. mesenteroides* NRRL B-1355 (Arguello-Morales *et al.*, 2000).

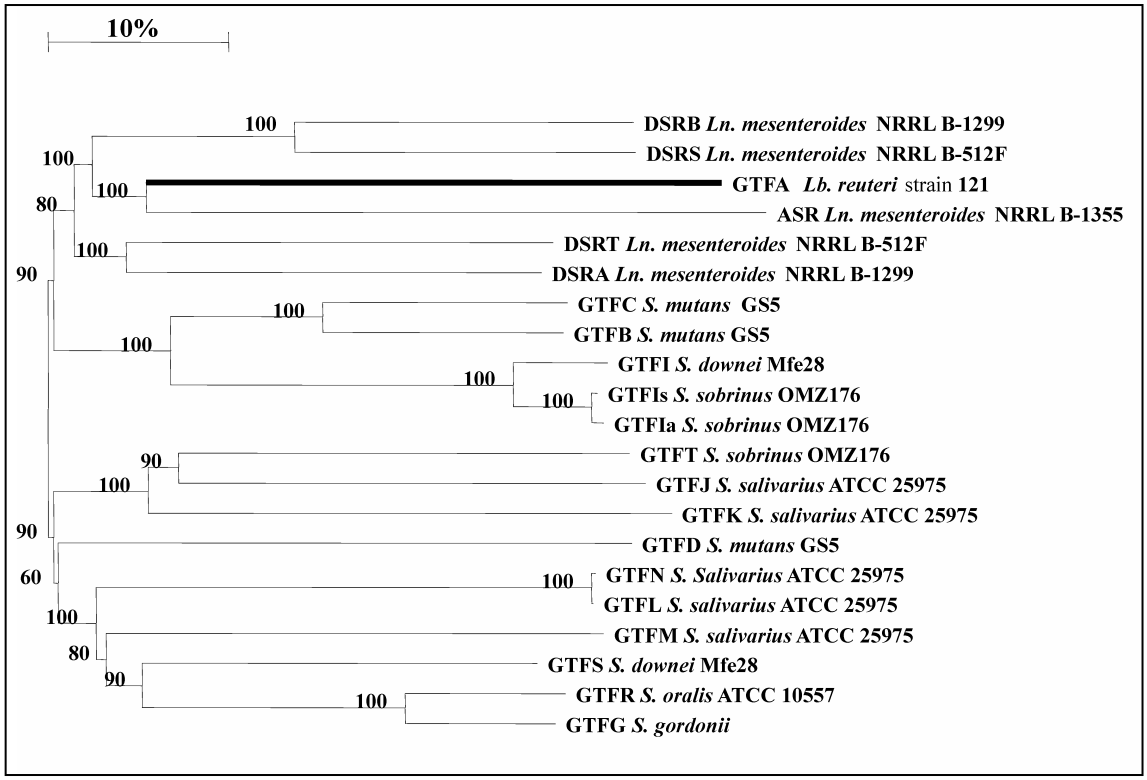

Figure 7. Dendrogram of glucosyltransferases of lactic acid bacteria. The horizontal distances are a measure for the differences at the amino acid level. The length of the upper bar indicates 10% difference. Bootstrap values are given at the root of each branch (in percentages). GTFA of *Lb. reuteri* strain 121 is indicated with a bold line.

Analysis of the glucans produced by *Lb. reuteri* and *E. coli* containing p15gtf

Extracts of *E. coli* (p15gtf) cells and supernatants of sucrose grown cultures of *Lb. reuteri* incubated with sucrose both produced high molecular weight glucans. Using HPSEC-MALLS, the average molecular weight of the glucan produced by *Lb. reuteri* strain 35-5 was determined as 4.10^7 Da (± 5%), whereas that of the glucan produced by *E. coli* harboring p15gtf was 8.10^7 Da (± 5%).

	Lb. reuteri strain **121**	*Lb. reuteri* **strain 35-5**	*E. coli* **(p15GTF)**
Linkage type		**Methylation (%)**	
Terminal	24	25	21
α-(1→4)	42	43	44
α-(1→6)	22	21	24
α-(1→4,6)	12	11	11

Table 1. Methylation analysis of the glucans produced by *Lb. reuteri* strains and *E. coli* GTFA.

The ^{1}H-NMR spectra of the glucans produced by the recombinant GTF present in *E. coli* and the GTF enzyme in *Lb. reuteri* strain 35-5, producing only the glucan, were virtually identical (Fig. 8). Comparison of both ^{1}H-NMR spectra with that of potato starch (Gidley, 1985) showed that both glucans consist of α-(1→4) and α-(1→6) linked glucopyranosyl units. The identical nature of the glucans was confirmed by methylation analysis (Table 1).

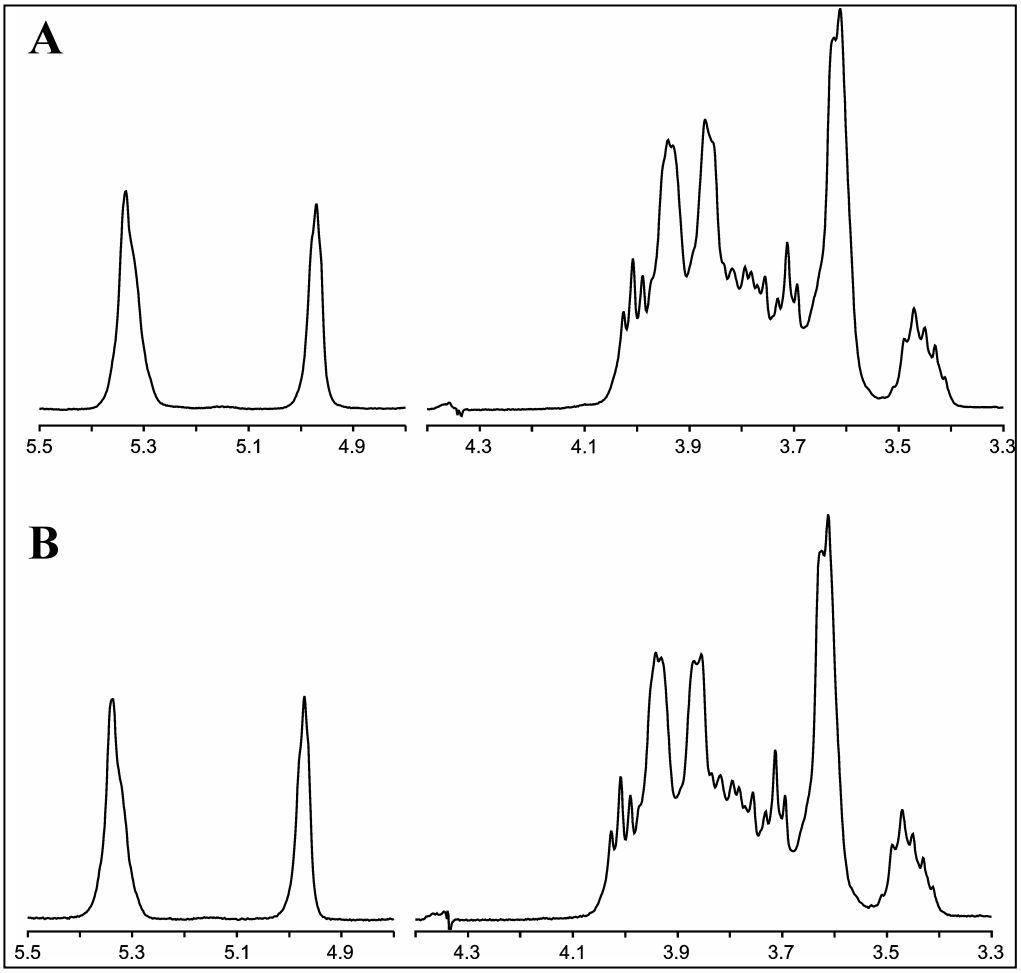

Figure 8. 500-MHz ^{1}H-NMR spectra of the glucan produced by *Lb. reuteri* strain 35-5 GTFA present in culture supernatant (**A**) and by *E. coli* GTFA cell extracts (**B**), recorded in D_2O at 80°C. Chemical shifts are given in parts per million relative to the signal of internal acetone (δ=2.225).

DISCUSSION

This paper reports the molecular characterization of the first *Lactobacillus* gene (*gtfA*) encoding a glucosyltransferase enzyme (GTFA). A detailed analysis showed that GTFA produces a unique soluble glucan in which the majority of the linkages are of the α-(1→4) glucosidic type. The glucan also contains α-(1→6) linked glucosyl units and 4,6-disubstituted α-glucosyl units at the branching points. Expression of *gtfA* in *E. coli* yielded an active glucosyltransferase, synthesizing the same highly branched soluble glucan.

GTFA showed high similarity with streptococcal and *Leuconostoc* glucosyltransferases. Similar to other glucosyltransferases, GTFA contains an N-terminal signal sequence, a variable N-terminal domain, a catalytic core and a C-terminal domain. Striking features of GTFA are its relatively large variable domain (702 amino acids) containing 5 distinct unique repeats (RDV repeats, Fig. 4), and its relatively short C-terminal domain (267 amino acids). Alternansucrase of *Ln. mesenteroides* NRRL B-1355 also possesses three N-terminal repeats (Janecek *et al.*, 2000) but these do not show homology to the N-terminal repeats found in GTFA. The exact function of the variable domain is unknown. The variable domain of GTFI from *Streptococcus downei* Mfe28 contains no repeats and is five times smaller than the GTFA domain. Deletion of the GTFI variable domain yielded a mutant enzyme which retained function (Monchois *et al.*, 1999a).

Based on alignments with other glucosyltransferases from lactic acid bacteria (Monchois *et al.*, 1999d), putative catalytic residues in *Lb. reuteri* strain 121 GTFA are Asp^{1024}, Glu^{1061} and, Asp^{1133}. The putative calcium-binding site is Asp^{986}, the putative chloride binding site Arg^{1022}. Addition of Ca^{2+} increases enzyme activity and stability (data not shown). Five residues may play a role in the binding of acceptor molecules and the transfer of the glucosyl residue. These are GTFA residues $Asp^{1027,}$, Asn^{1028}, Asp^{1062} and Trp^{1063} (Fig. 5). The fifth amino acid, possibly playing a role in acceptor binding or transfer of the glucosyl residue, a Ser in other glucosyltransferases (except for ASR of *Ln. mesenteroides* and DSRA of *Ln. mesenteroides*), was replaced by Asn^{1064} in GTFA.

The C-terminal domain of GTFA, consisting of 267 amino acids, is shorter than corresponding domains in other glucosyltransferases (~500 amino acids). The C-terminal domain of streptococcal and *Leuconostoc* glucosyltransferases consist of a series of different tandem repeats, which have been divided into four classes: A, B, C and D repeats. These repeats exhibit high similarity to the repeats found in the glucan binding protein from *S. mutans* as well as the ligand binding domains in *Clostridium difficile* toxin A and the lysins from *S. pneumoniae* (Giffard *et al.*, 1993, Wren, 1991). DSRS from *Ln. mesenteroides* NRRL-512F contains in addition to A and C repeats also N repeats, which have not been identified in streptococcal GTFs. Alternansucrase from *Ln. mesenteroides*

NRRL B-1355 contains a single A repeat and distinct short repeats $DG(X)_4APY$ (Janecek *et al.*, 2000). Within the A-D repeats a repeating unit designated YG can be distinguished (Giffard & Jacques, 1994). The A, B, C and D repeats present in distinct patterns in the C-terminal domain of other glucosyltransferases were not found in GTFA. Instead four YG-repeating units and seven less conserved YG-repeats could be identified (Fig. 6).

Highest overall homology of GTFA at the amino acid level was found with alternansucrase from *Ln. mesenteroides* NRRL B-1355 (Arguello-Morales *et al.*, 2000), responsible for the synthesis of an alternan with 50% α-(1→6) and 50% α-(1→3) linkages, and with DSRS from *Ln. mesenteroides* NRRL B-512F (Wilke-Douglas *et al.*, 1989), synthesizing a dextran with 95% α-(1→6) and 5% α-(1→3) linkages. Homology of GTFA with other glucosyltransferases was highest in the highly conserved putative catalytic domain, which had roughly the same size and structure as the corresponding domains of other glucosyltransferases. However, not all the conserved residues were found in the *Lb. reuteri* strain 121 GTFA. Relatively many differences with amino acids conserved in other glucosyltransferases were found directly downstream of the putative catalytic Asp^{1024} (Fig. 5). This region constitutes the α/β barrel 4 of the enzymes of family 13 of glycoside hydrolases (MacGregor *et al.*, 1996). The domain directly downstream of the catalytic Asp^{1024} contains the conserved amino acids Asp-Ala-Val-Asp-Asn in other glucosyltransferases. In CGTase these residus constitute part of the acceptor-binding site (residues Asp^{229}-Ala-Val-Lys-His^{233}, in *Bacillus circulans* 251 CGTase), responsible for the stereospecific positioning of the molecule accepting the glucosyl unit (Knegtel *et al.*, 1995). The structure of this acceptor site determines the type of glucosidic bond formed (Uitdehaag *et al.*, 1999). In the corresponding region of GTFA, Pro^{1026} is found in a position where a conserved Val is found in other glucosyltransferases (Fig. 5). Compared with Val, the presence of Pro causes a more rigid protein structure, which may have a direct effect on the type of glucosidic bonds formed in the glucan synthesized by the enzyme. The presence of the Pro^{1026} residue could therefore be part of the explanation for the unique structure of the glucan with α-(1→4) and α-(1→6) bonds, produced by GTFA. The conserved Val is present in amylosucrase (Fig. 5), a glucosyltransferase synthesizing α-(1→4) bonds. However, immediately downstream of this Val, the conserved Asp-Asn residues are replaced by Ala-Phe (De Montalk *et al.*, 1999). The following amino acid in amylosucrase is Ile, which is also present at that position in GTFA, whereas in other glucosyltransferases a conserved Val is found (Fig. 5). This also suggests that the above mentioned region downstream of the catalytic Asp^{1024}, may be of influence on the type of bonds being formed. Therefore, Pro^{1026} and Ile^{1029} of GTFA are likely targets for site directed mutagenesis experiments.

The partial open reading frame upstream of *gtfA* (ORF2, Fig. 1) may encode a second GTF enzyme in *Lb. reuteri* strain 121. However, the N-terminal amino acid sequence of glucosyltransferase purified from culture supernatants of *Lb. reuteri* strain 121 was the

same as the deduced N-terminal amino acid sequence of the *gtfA* gene, and the M_r and pI of the purified enzyme were the same as predicted from the nucleotide sequence of the *gtfA* gene. Furthermore, ^{1}H-NMR spectra of the glucans produced by the *Lb. reuteri* GTFA present in culture supernatant and by the *E. coli* GTFA in cell extracts were virtually identical (Fig. 8). This, combined with the results of the methylation (Table 1), and the molecular weight determinations of the glucans, shows that the *E. coli* GTFA and the *Lb. reuteri* enzyme present in culture supernatants synthesize the same glucan with a unique structure: a highly branched glucan containing α-(1→4) and α-(1→6) bonds. Therefore it is concluded that the *gtfA* gene encodes the active glucosyltransferase of *Lb. reuteri* strain 121.

In future, ORF2 upstream of *gtfA* will be characterized in further detail and GTFA will be characterized molecularly and biochemically.

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Chapter 3

Biochemical and molecular characterization of *Lactobacillus reuteri* 121 reuteransucrase

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SUMMARY

Lactobacillus reuteri strain 121 uses sucrose for synthesis of a unique, soluble glucan ("reuteran") with mainly α-(1→4) glucosidic linkages. Previously, the gene (*gtfA*) encoding this glucansucrase enzyme has been characterized (Kralj *et al.*, 2002). Here a detailed biochemical and molecular analysis of this GTFA enzyme is presented. This is believed to be the first report describing the reuteransucrase enzyme kinetics and the oligosaccharides synthesized with various acceptors.

Alignments of the GTFA sequence with glucansucrases from *Streptococcus* and *Leuconostoc* origin served to identify conserved amino acid residues in the catalytic core critical for enzyme activity. Mutants Asp1024Asn, Glu1061Gln and Asp1133Asn displayed 300-1,000 fold reduced specific activities.

To investigate the role of its relatively large N-terminal variable domain (702 amino acids) and its relatively short C-terminal (putative) glucan binding domain (267 amino acids, with 11 YG-repeats), various truncated derivatives of GTFA (1781 amino acids) were constructed and characterized. Deletion of the complete N-terminal variable domain of GTFA (GTFA-ΔN) had little effect on reuteran characteristics (size, distribution of glucosidic linkages), but the initial transferase activity of the mutant enzyme increased drastically. Sequential C-terminal deletions (up to 6-YG repeats) in GTFA-ΔN also had little effect on reuteran characteristics. However, enzyme kinetics drastically changed. Deletion of 7, 8 or 11 YG-repeats resulted in dramatic loss of total enzyme activity (43×, 63× and 1,000× fold reduced specific activities, respectively). Characterization of sequential C-terminal deletion mutants of GTFA-ΔN revealed that the C-terminal domain of reuteransucrase has an important role in glucan binding.

INTRODUCTION

Reuteransucrase (GTFA) from *Lactobacillus reuteri* 121 is a 1,781 amino acid glucosyltransferase (GTF) enzyme (EC 2.4.1.5; common name glucansucrase), that synthesizes a unique soluble glucan polymer (reuteran) with mainly α-(1→4) glucosidic linkages and significant amounts of α-(1→6) and α-(1→4,6) glucosidic linkages. (van Geel-Schutten *et al.*, 1999, Kralj *et al.*, 2002). Two different reactions are catalyzed by glucansucrase enzymes, depending on the nature of the acceptor: i) hydrolysis, when water is used as acceptor; ii) glucosyl transfer (transferase), which can be divided in: a) polymerization, when the growing glucan chain is used as acceptor, and b) oligosaccharide synthesis, when oligosaccharides (e.g. maltose, isomaltose) are used as acceptor. Where studied, the linkage specificity of glucansucrases is conserved in oligosaccharide synthesis (Dols *et al.*, 1997, Cote & Robyt, 1982, Robyt & Walseth,

1978), and oligosaccharides are elongated at their non-reducing end (Dols *et al.*, 1997, Arguello Morales *et al.*, 2001, Monchois *et al.*, 2000a, Mukasa *et al.*, 2000). A biochemical characterization of the reactions catalyzed by GTFA of *Lb. reuteri* 121 remained to be carried out.

GTF proteins of lactic acid bacteria share a common structure and are composed of four distinct domains: their N-terminal end starts with (i) a signal peptide of 32-34 amino acids, followed by (ii) a highly variable stretch of 123-129 amino acids, (iii) a highly conserved catalytic or sucrose binding domain of about 1,000 amino acids and (iv) a C-terminal domain of about 500 amino acids, composed of a series of tandem repeats (Monchois *et al.*, 1999d). We are interested in the precise roles of these various domains and repeats in the overall functioning of GTF enzymes.

Amino acid sequence comparisons revealed that the catalytic core of GTF proteins is constituted of 8 β-sheets alternated with 8 α-helices (a $(\beta/\alpha)_8$ barrel structure) similar to glycoside hydrolases of family 13 (α-amylase family). This family includes for instance α-amylase and cyclodextrin glycosyltransferase (CGTase) (van der Veen *et al.*, 2000). In GTFs, however, this $(\beta/\alpha)_8$ barrel structure is circularly permuted (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997). Therefore GTFs are classified in family 70 of glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY/index.html).

Whereas for the α-amylase family the catalytic mechanism is known (McCarter & Withers, 1994, Uitdehaag *et al.*, 1999), the exact catalytic mechanism of GTF enzymes remains to be elucidated (Monchois *et al.*, 1999d). Amino acid residues crucial for catalysis in glucansucrases of family 70 have been identified as Asp453 (putative catalytic nucleophile), Glu491 (putative acid/base catalyst) and Asp564 (putative transition state stabilizer) in GTFI from *Streptococcus downei* Mfe28 (Fig. 1) (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997).

The equivalent invariable residues, in enzymes of α-amylase family 13, are Asp229, Glu257 and Asp328 (CGTase *Bacillus circulans* 251 numbering). In enzymes of both families the first mentioned Asp residue is involved in formation of the covalent glucosyl-enzyme complexes (Mooser *et al.*, 1991, MacGregor *et al.*, 1996, Uitdehaag *et al.*, 1999). The importance of this Asp residue has also been shown for other GTFs by site-directed mutagenesis experiments (Kato *et al.*, 1992, Monchois *et al.*, 1997, Devulapalle *et al.*, 1997) (Fig. 1). Based on alignments with glucansucrases from lactic acid bacteria (Monchois *et al.*, 1999d), putative catalytic residues in *Lb. reuteri* strain 121 GTFA were identified and mutated (see results, Fig. 1).

Different repeating units have been identified in the N-terminal variable domains of several glucansucrases: A-repeats in alternansucrase and dextransucrases from *Leuconostoc mesenteroides* sp. (Janecek *et al.*, 2000), motif T in DSRT from *Ln. mesenteroides* NRRL B-512F (Funane *et al.*, 2000), and motif S in DSRE from *Ln. mesenteroides* NRRL B-1299 (Bozonnet *et al.*, 2002). The function of the N-terminal

variable domain (and these repeats) has remained unclear. Deletion studies showed that it does not play a significant role in glucansucrase activity (Abo *et al.*, 1991, Monchois *et al.*, 1999a).

Bacterial Strain	Main α-linkages in glucan polymer			A		B		C
				∀		⇓		♦
Lb. reuteri 121	1→4 / 1→6	GTFA	1016	ANFDSVRV***D***APDNIDADLMNI	1056	HINIL***E***DWNHADPEY	1126	YSFVRAH***D***NNSQDQI
S. mutans GS5	1→3	GTFB	443	ANFDSIRV**D**AVDNVDADLLQI	484	HLSILEAWSDNDTPY	555	YSFIRAHDSEVQDLI
S. mutans GS5	1→6	GTFD	457	ANFDGVRVDAVDNVNADLLQI	498	HLSILEAWSDNDPQY	577	YIFIRAHDSEVQTVI
S. downei Mfe28	1→3	GTFI	445	ANFDSIRV**D**AVDNVDADLLQI	486	HVSIV**E**AWSDNDTPY	557	YSFARAH**D**SEVQDLI
S. salivarius ATCC 25975	1→3	GTFJ	463	ANFDGIRVDAVDNVDADMLQL	504	HISVLEAWSLNDNHY	605	YVFIRAHDNNVQDII
S. salivarius ATCC 25975	1→6	GTFK	453	AHFDGIRVDAVDNVSVDMLQL	494	NISILEAWSHNDPYY	575	YLFVRAHDSEVQTVI
S. downei MFe28	1→6	GTFS	388	ANFDGVRVDAVDNVNADLLQI	429	HLSILEAWSGNDNDY	470	YVFIRAHDSEVQTRI
Ln. mesenteroides NRRL B-1299	1→6	DSRB	525	ANFDGIRVDAVDNVDADLLQI	566	HLSILEDWSHNDPEY	637	YSFVRAHDSEVQTVI
Ln. mesenteroides NRRL B-512F	1→6	DSRS	543	ANFDGIRV**D**AVDNVDADLLQI	584	HLSILEDWSHNDPLY	655	YSFVRAHDSEVQTVI
Ln. mesenteroides NRRL B-1355	1→6 / 1→3	ASR	626	ANFDGIRVDAVDNVDADLLKI	667	HLSILEDWNGKDPQY	759	YSFVRAHDYDAQDPI
				*:**.:**** **:..*::::		::.::* *. * *		* * **** : * *

Figure 1. Amino acid sequence alignment of highly conserved stretches (A, B, C) in catalytic domains of dextran-, mutan-, alternan and reuteransucrases of lactic acid bacteria (also see (Monchois *et al.*, 1999d). GTFA, *Lb. reuteri* 121 (Kralj *et al.*, 2002); GTFB, *S. mutans* GS5 (Shiroza *et al.*, 1987); GTFD, *S. mutans* GS5 (Honda *et al.*, 1990); GTFS, *S. downei* Mfe28 (Gilmore *et al.*, 1990); GTFI, *S. downei* Mfe28 (Ferretti *et al.*, 1987); GTFJ, *Streptococcus salivarius* ATCC 25975 (Giffard *et al.*, 1991); GTFK, *S. salivarius* ATCC 25975 (Giffard *et al.*, 1993); DSRB, *Ln. mesenteroides* NRRL B-1299 (Monchois *et al.*, 1998a); DSRS, *Ln. mesenteroides* NRRL B-512F (Monchois *et al.*, 1997); ASR, *Ln. mesenteroides* NRRL B-1355 (Arguello-Morales *et al.*, 2000); ***** , identical residue; **:** , highly conserved residue; **.** , conserved residue; ∀, putative nucleophile(MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); ⇓, putative acid/base catalyst (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); ♦, putative residue stabilizing the transition state (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); **A**, catalytic amino acids which have been mutated in other studies (Monchois *et al.*, 1999d); ***A***, catalytic amino acids of GTFA of *Lb. reuteri* 121 identified and characterized in this study.

The C-terminal domains of *Streptococcus* and *Leuconostoc* GTF enzymes consist of a series of different tandem repeats, which have been divided into four classes: A, B, C and D repeats. DSRS from *Ln. mesenteroides* NRRL-512F contains in addition to A and C repeats also N repeats (Monchois *et al.*, 1998b), which have not been identified in other GTFs. Alternansucrase from *Ln. mesenteroides* NRRL-B 1355 contains a single A repeat and distinct short repeats $DG(X)_4APY$ (Janecek *et al.*, 2000).Within the A-D repeats, a repeating unit designated YG can be distinguished (Giffard & Jacques, 1994). The C-terminal repeats play an important role in glucan binding (Abo *et al.*, 1991, Shah & Russell, 2002, Lis *et al.*, 1995, Monchois *et al.*, 1998b).

The structure of GTFA of *Lb. reuteri* 121 is unusual because the above-mentioned repeats are not present in its N- and C-termini. Instead, GTFA possesses a relatively large N-terminal variable domain (702 amino acids) with 5 RDV repeats. Its relatively short C-terminal domain (267 amino acids) contains 11 YG-repeats only (Fig. 2A) (Kralj *et al.*,

2002). This raised questions about the precise role of the N- and C-terminal domains in GTFA.

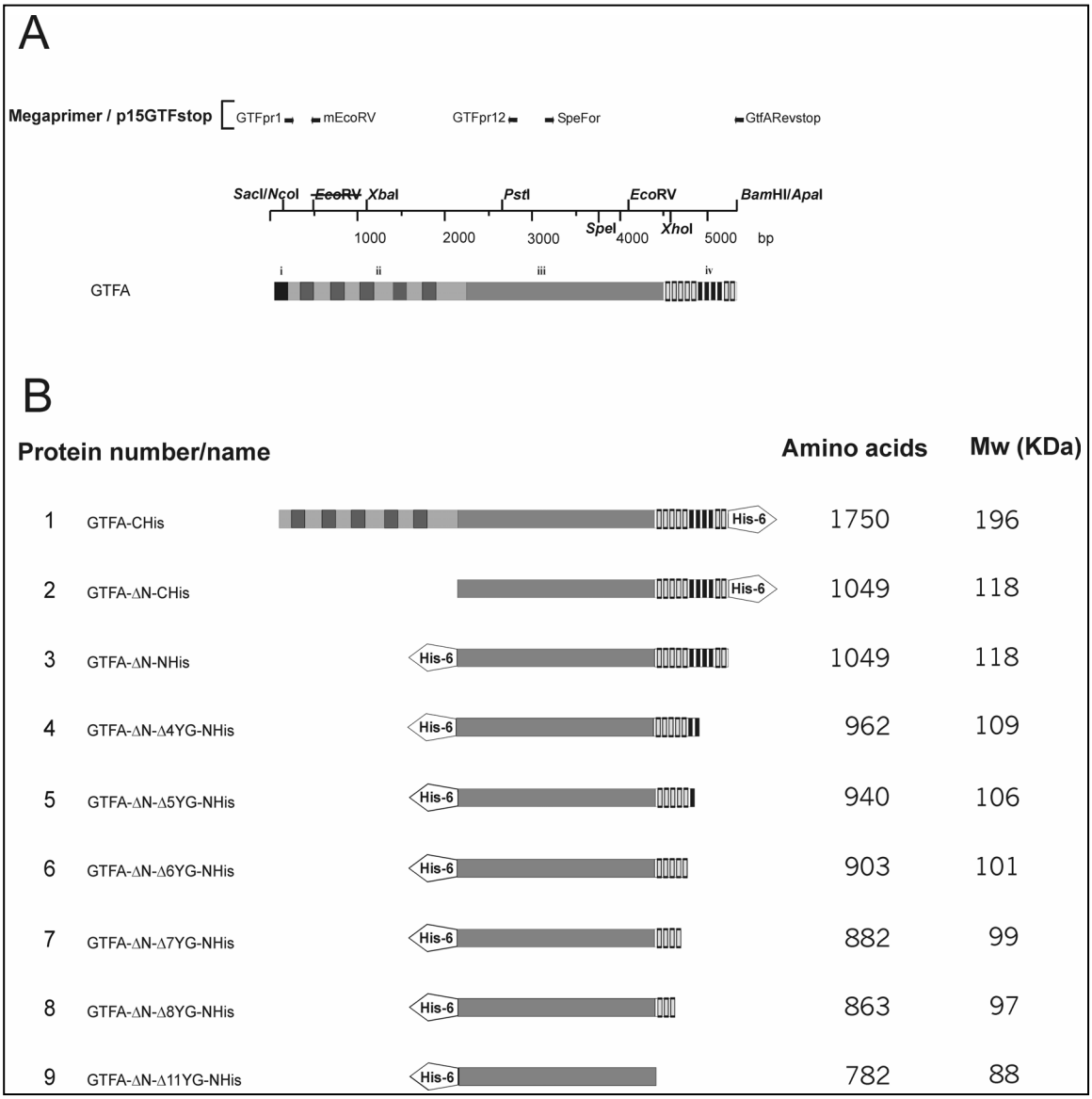

Figure 2. Overview of primers and restriction sites used for cloning, expression and production of *Lb. reuteri* 121 GTFA protein and (deletion) mutant derivatives in *E. coli* and domain organization of GTFA and (deletion) mutant derivatives. (**A**) Overview of primers used for the Megaprimer method and the construction of p15GTFstop. The different domains shown in GTFA are i) N-terminal signal sequence (not present in mutants); ii) variable region with 5 RDV repeats (dark grey boxes); iii) catalytic domain; iv) C-terminal (putative) glucan binding domain with 4 YG-repeating units (dark grey boxes) according to the definition of (Giffard & Jacques, 1994) and 7 less conserved YG-repeating units (light grey boxes). (**B**) Schematic representation of the domain structure of GTFA-CHis and the truncated derivatives constructed. Molecular sizes, number of amino acids and position of the His tag of the different protein constructs are indicated.

Here we describe the first molecular (construction of site-directed and deletions mutants) and biochemical (analysis of the main reactions catalyzed by wild type and mutant enzymes) characterization of an α-(1→4) synthesizing glucansucrase. Furthermore, we provide evidence for involvement of the C-terminal YG-repeats in glucan binding.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions

Escherichia coli DH5α (Phabagen) (Hanahan, 1983), *E. coli* TOP 10 (Invitrogen) and plasmid pBluescript II SK^+ (Stratagene) were used for cloning purposes. Plasmid pET15b (Novagen) was used for expression of the (mutant) *gtfA* gene(s) in *E. coli* BL21 Star (DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium (Ausubel *et al.*, 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (100 μg ml^{-1} ampicillin). Agar plates were made by adding 1.5 % agar to the LB medium.

Molecular techniques

General procedures for cloning, *E. coli* transformations, DNA manipulations, and agarose gel electrophoresis were as described (Sambrook *et al.*, 1989). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the enzyme suppliers (New England Biolabs; Roche Biochemicals). Primers were obtained from Eurogentec, Seraing, Belgium. Cycle sequencing (Murray, 1989) was performed on double stranded DNA using the Thermo Sequence fluorescent primer cycle sequence kit (Amersham Pharmacia Biotech). Sequence reactions were performed on the Amersham ALF-Express sequencing machine at the BioMedical Technology Center (Groningen, The Netherlands). DNA was amplified by PCR on a DNA Thermal Cycler PTC-200 (MJ Research) using *Pwo* DNA polymerase (Roche Biochemicals). Fragments were isolated from agarose gels using a Qiagen gel extraction kit (Qiagen) following the instructions of the supplier.

Plasmid construction

Plasmid pBGTF1, pBluescript carrying the Lb. reuteri 121 gene encoding GTFA (Kralj et al., 2002), without signal sequence and fused at the C-terminus with a stretch of six histidine residues (1,750 amino acids), was used. To facilitate future mutagenesis and nucleotide sequencing, the first of two EcoRV restriction sites (492 bp, 4105bp) was altered in pBGTF1, using the megaprimer method (Sarkar & Sommer, 1990) and the following primers: GTFpr1, 5'-GATGCATGAGCTC**CCATGG**ACCAACAAGTTCAG

CAAGCTTCC-3'**,** containing *Sac*I (italics) and *Nco*I (bold) restriction sites, and mEcoRV, 5'-GGTTGTGAT**G**TCACGCACAATGATTTG-3'**,** containing a mutated EcoRV site (underlined, silent mutation by change of base shown in bold face). In a subsequent PCR reaction the amplified product (~500 bp) was used as (forward) primer together with GTFpr12 5'-GTGCATTAAAGTACGTAACCAATCAGTATTTCCGG-3', 1600 bp downstream of a *Xba*I restriction site (Fig. 2A). The resulting product of 2600 bp was digested with *Sac*I and *Xba*I and ligated in the corresponding sites of pBGTF1, yielding pBGTF2.

Plasmid pBGTF2 now consists (from 5' to 3') of four cassettes of 1000-1500 bp: i) *Sac*I&(*Nco*I)/*Xba*I (1001 bp), ii) *Xba*I/*Pst*I (1546 bp), iii) *Pst*I/*Eco*RV (1454 bp), and iv) *Eco*RV/(*Bam*HI)&*Apa*I (1269 bp) (Fig 2A). This plasmid was digested with *Pst*I (2655 bp) and *Eco*RV (4105 bp) and the resulting fragment (1454 bp) was ligated in the corresponding sites of pBluescript II SK^+ (Stratagene), yielding pBPE1500. This (small) construct was used for site-directed mutagenesis, sequencing and rapid exchange (using *Pst*I and *Eco*RV restriction sites) with pBGTF2 (see below). pBGTF2 was also digested with *Nco*I/*Bam*HI (5257 bp) and ligated in the corresponding sites of pET15b (Novagen), yielding p15GTF2 (encoding GTFA-CHis, Fig. 2B). This construct was used in subsequent cloning steps.

In order to create a construct without C-terminal His-tag, but with a stop codon, the following primers were used to exchange the C-terminal part of p15GTF2: SpeFor: 5'-GGGCTTTCAGATGCAACTAATCGTTGGGG-3', lying 400 bp upstream of a *Spe*I restriction site, and GtfARevstop 5'-TCGAT*GGGCCC*C**GGATCC**TATTATAGTTTAT TTTGATCAAGCATCTTACC-3', containing *Bam*HI (bold) and *Apa*I (italics) restriction sites (Fig. 2A). The resulting PCR product (~2000 bp) was digested with *Spe*I and *Bam*HI (1592 bp) and ligated in the corresponding sites of p15GTF2 (see above), yielding p15GTFstop. In all cases successful mutagenesis was confirmed by nucleotide sequencing.

Site-directed mutagenesis of putative catalytic residues of GTFA

Plasmid pBPE1500, (see above), was used as template for mutagenesis. The QuickChange™ site-directed mutagenesis kit (Stratagene) was used to construct mutants D1024N, E1061Q and D1133N, using different primer pairs (Table 1A). Successful mutagenesis resulted in the appearance or disappearance of restriction sites, allowing rapid screening of potential mutants. After successful mutagenesis (confirmed by nucleotide sequencing), pBPE1500 (containing mutation) was digested with *Pst*I and *Eco*RV and ligated in the corresponding sites of pBGTF2. The resulting plasmid pBGTF2 (containing mutation) was digested with *Nco*I/*Bam*HI and the resulting 5.3 kb fragment was ligated into the corresponding sites of the expression vector pET15b (Novagen) yielding p15GTF2 containing mutation.

Construction of truncated *gtfA* genes

Appropriate primer pairs and template DNA was used (Table 1B) to construct eight different deletion mutants of *gtfA* (Fig. 2B). Deletion mutants were constructed by exchange of restriction fragments and checked by nucleotide sequencing.

Table 1. Oligonucleotides used for site-directed (**A**) and deletion mutagenesis (**B**) of *gtfA*. Nucleotides in bold type/italics represent mismatches with the sequence of *gtfA*. Nucleotides underlined represent introduced or removed (in the case of mutation E1061Q) restriction sites. H = histidine residue, st = stop codon.

Mutation	Forward primer (5'→3')	Reverse primer (5'→3')	Restriction sites and plasmids used to exchange fragments
A			
D1024N	GATTCTGTACGGGT***TAAC***GCACCGG *HpaI*	CCGGTGC***GTTA***ACCCGTACAGAATC *HpaI*	*Pst*I/*Eco*RV (pBGTF2)
E1061Q	GCATAT***A***AATATTCTT***C***AAGACTGGAATC (*Ase*I)	GATTCCAGTCTT***G***AAGAATATT***T***ATATGC (*Ase*I)	*Pst*I/*Eco*RV (pBGTF2)
D1133N	CGTTCGGGGC***C***CAC***A***AC***C***AATAATTCTC *ApaI*	GAGAATTATT***G***T***T***GTG***G***GCCCGAACG *ApaI*	*Pst*I/*Eco*RV (pBGTF2)
B			
GTFA-ΔN-CHis	GATGCATGAGCTCCCATGGGCATTAACGGTCAACAA *Sac*I *Nco*I ACCCTATTATATTG	GAGCATCTTGGTTATCACTTGCTCCAACTGG	*Nco*I/*Spe*I (p15GTF2)
GTFA-ΔN-NHis	GATGCATGAGCTCCCATGGGCCATCACCATCACCATCAC *Sac*I *Nco*I **H H H H H H** ATTAACGGTCAACAATATTATATTGACCC	GAGCATCTTGGTTATCACTTGCTCCAACTGG	*Nco*I/*Spe*I (p15GTFstop)
GTFA-ΔNΔ4YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTAAACGAAA *ApaI* *Bam*HI **st st** TGTTTATTTTCAACCATCTTACC	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ5YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTATTCAATA *ApaI* *Bam*HI **st st** AAGGTATTCTTAACCATATTACC	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ6YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTAAATTGGC *ApaI* *Bam*HI **st st** AAGAATTGCTTTGTAGTAGTACC	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ7YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTATACATAA *ApaI* *Bam*HI **st st** CCAGAACCACGATGGAGAATGTTTG	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ8YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTATTCAGTT *ApaI* *Bam*HI **st st** ATCTTTTGTGAAGGATCAATAGC	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ11YG-NHis	GGGCTTTCAGATGCAACTAATCGTTGGGG	ATATCGATGGGCCCCGGATCCTATTATTGGTCAG *ApaI* *Bam*HI **st st** GAACCCAATCCGCCATTAC	*Spe*I/*Bam*HI (p15GTFA-ΔN-NHis)

Purification of GTFA (mutant) proteins

Cells of *E. coli* BL21star (DE3) harboring the different pET15b derivatives were harvested by centrifugation (10 min at 4 °C at 10,000 × *g*) after 16 h of growth at 37 °C (without induction). The pellet was washed with 50 mM phosphate buffer pH 8.0. Pelleted cells were resuspended in 50 mM sodium phosphate buffer pH 8.0 containing 250 mM NaCl, 5 mM β-mercaptoethanol and 10 mM imidazole. Cells were broken by sonication (7 × 15 sec at 7 micron with 30 sec intervals) and centrifuged (10 min at 4 °C at 10,000 × *g*). The clear lysate containing GTF activity was loaded on a Ni-NTA column

(Qiagen). Binding was realized using 50 mM sodium phosphate buffer pH 8.0 containing 250 mM NaCl, 5 mM β-mercaptoethanol and 10 mM imidazole, followed by washing using the same buffer. Elution of His-tagged protein(s) was performed using 50 mM sodium phosphate buffer pH 8.0 with 250 mM NaCl, 1 mM β-mercaptoethanol and 200 mM imidazole. Eluted proteins were desalted with 20 mM Tris buffer, pH 8.0, using a 5 ml Hi-Trap desalting column (Amersham Pharmacia Biotech). Subsequently, the samples were purified on an ACTA prime FPLC system (Amersham Pharmacia Biotech), using a 1 ml Resource-Q column (Amersham Pharmacia Biotech) and a linear gradient of 30 ml with 1 M NaCl in 20 mM Tris buffer pH 8.0 as eluens at a flow rate of 1 $ml.min^{-1}$. Proteins present in the elution peak were desalted with 25 mM sodium acetate buffer pH 4.7, supplemented with 1 mM $CaCl_2$, using a 5 ml Hi-Trap desalting column (Amersham Pharmacia Biotech). At each stage of the purification, the GTF transferase activity was quantified as previously described (van Geel-Schutten *et al.*, 1999). The degree of purity of the different mutants was determined by SDS-PAGE (Laemmli, 1970).

Protein concentrations were determined using the Bradford method using the Bio-Rad reagent and BSA (bovine serum albumin) as a standard (Bio-Rad). From 250 ml cultures of *E. coli* strains expressing full-length (wild type or mutant) and N-or C-terminally truncated protein, approximately 0.25 and 1 mg of highly purified protein was obtained, respectively.

Glucan binding assay

The glucan binding ability of GTFA (deletion mutants) was measured as described previously (Lis *et al.*, 1995), and as improved by (Shah & Russell, 2002). Briefly, 30 pmol of GTFA (mutant) protein in 200 µl PBS-T (20 mM sodium phosphate pH 7.3, 150 mM NaCl, 0.05% tween 20) was incubated overnight at 4°C in Ni-NTA coated HisSorb plates (Qiagen). Proteins containing a His-tag are bound to the Ni-NTA, non-binding protein was removed by washing with PBS-T (four times 1 min). After incubation for 20 min with 200 µl biotin labelled dextran (100 µg/µl; Fluka) in PBS-B (20 mM sodium phosphate pH 7.3, 150 mM NaCl, 0.2% BSA), which may bind to the glucan-binding domain (GBD) of the protein, washing was performed with PBS-T, as described before. Extravidin-alkaline phosphatase conjugate (1/10,000; Sigma) in PBS-B, which binds to the biotin part of the labelled dextran, was added and incubated for 1 h, followed by washing with PBS-T. Subsequently, alkaline phosphatase substrate 4-nitrophenyl phosphate (100 µl; Roche Biochemicals) was added, yielding a yellow color upon its hydrolysis by the bound alkaline phosphatase. The colour change was monitored using a SpectraMax Plus 384 plate reader (Molecular Devices).

Enzyme activity assays

The various reuteransucrase activities were determined by measuring glucose and fructose release (enzymatically) from sucrose conversion (van Geel-Schutten *et al.*, 1999). The amount of fructose released (V_F) corresponds to total enzyme activity (initial formation of leucrose or other sucrose isomers was negligible, see results and data not shown). The amount of free glucose (V_G) represents the hydrolytic activity of the enzyme. The amount of fructose minus the amount of free glucose reflects the transferase activity (V_F-V_G). Unless indicated otherwise, reactions were performed at 50 °C in 25 mM NaAc buffer, pH 4.7, containing 1 mM $CaCl_2$ and 30 nM purified (mutant) reuteransucrase enzyme. One unit of enzyme activity is defined as the release of 1 µmol of monosaccharide per min. In case of very low activity of mutant proteins, assay conditions were modified as follows: reactions were performed with 25 × more protein, and aliquots were removed at 30 min intervals instead of 1 min.

(i) Kinetic parameters. Kinetic assays were performed using twenty-four different sucrose concentrations ranging from 0.25 to 100 mM. Over a 6 min incubation period, samples of 25 µl were withdrawn every minute and inactivated with 2.5 µl 1 M NaOH. Curve fitting of the data was performed with the "SigmaPlot" program (version 8.0) using either, the Michaelis-Menten formula [$y = (a \times x) / (b + x)$], or the same formula with a substrate inhibition constant [$y = (a \times x) / (b + x + (x^2/c))$]. In these equations y is the specific activity ($U.mg^{-1}$), x is the substrate concentration (mM sucrose), a is the maximal reaction rate, Vmax ($U.mg^{-1}$), b is the affinity constant for the substrate (Km, mM sucrose), and c is the substrate inhibition constant (Ki, mM sucrose).

(ii) Effect of maltose on initial GTFA activity. The initial rate of oligosaccharide synthesis was examined by measuring the effect of maltose on (mutant) GTFA enzyme activity, using 50 mM sucrose and 100 mM maltose. The activity was determined by measuring fructose release.

Product analysis

(i) Product spectrum from sucrose. After complete depletion of sucrose (100 mM, 60 h at 50 °C), the concentrations of fructose, glucose and leucrose in the reaction medium of GTFA (mutants) were determined using anion exchange chromatography (see below). The amount of fructose released (97.9 %) and leucrose (2.1 %) synthesized from sucrose corresponds to 100 %. Subtracting the free glucose (23.1 %; due to hydrolysis) from the free fructose (97.9 %) concentration, allowed calculation of the yield of reuteran synthesis (74.8 %) from sucrose (data of GTFA-CHis used here as an example, see Table 4).

(ii) Oligosaccharide synthesis with maltose and isomaltose as acceptors. Oligosaccharide synthesis was analyzed using 100 mM sucrose together with maltose or isomaltose (100 mM each). After complete consumption of sucrose (60 h at 50 °C), samples were diluted 500-1000 times in a 90% DMSO solution. Maltose, isomaltose, maltotriose, panose (Sigma), isomaltotriose (TNO Nutrition and Food Research, Groningen, The Netherlands), sucrose (Acros Organics), fructose, glucose (Merck), and leucrose (Pfeiffer & Langen) were used as standards. The percentage of oligosaccharide synthesis from sucrose and acceptor was determined by subtracting the amount of unused acceptor from the initial acceptor concentration. Separation of oligosaccharides was achieved with a CarboPac PA1 anion exchange column (250 × 4 mm; Dionex) coupled to a CarboPac1 guard column (Dionex). The following gradient was used: eluent A (0 min, 100%); (5 min, 100%); (50 min, 92%); (55-58 min, 0%); (60 min, 100%); (75 min, 100%). Eluent A was sodium hydroxide (0.1 M) and eluent B was NaAc (0.6 M) in sodium hydroxide (0.1 M). Detection was performed with an ED40 Electrochemical detector (Dionex) with an Au working electrode and an Ag/AgCl reference electrode with a sensitivity of 300 nC. The pulse program used was: +1.0 Volt (0-0.40s); +0.7 Volt (0.41-0.60 s); -0.1 Volt (0.61-1.00 s). Data were integrated using a Turbochrom (Applied Biosystems) data integration system.

To determine the degree of polymerization (DP) of unknown oligosaccharides a BC-200 Ca^{2+} column (at 85 °C; 300 by 7.8 mm; Benson Polymeric) eluted with water (0.2 ml/min) was used. Detection was done by using a model 830-RI refractive index detector at 40 °C (Jasco).

In vitro glucan production by (mutant) GTFA enzymes and glucan structure analysis

(i) Polymer production. Purified (mutant) enzyme preparations were incubated overnight with 146 mM sucrose, using the conditions described above under enzyme assays. Glucans produced were isolated by precipitation with ethanol (van Geel-Schutten *et al.*, 1999).

(ii) Methylation analysis. Polysaccharides were permethylated using methyl iodide and Na-Dimsyl in DMSO at room temperature (Hakomori, 1964). After hydrolysis with 2 M trifluoroacetic acid (1 h, 125 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ (Harris *et al.*, 1984). Mixtures of partially methylated alditol acetates obtained were analyzed by GLC on a CP Sil 5 CB column (25 m × 0.53 mm; Chrompack) and by GLC-mass spectrometry (MS) on a RTX 5 Sil MS (30 m × 0.25 mm; Restek) column (Chaplin, 1982, Jansson *et al.*, 1976).

(iii) Molecular masses of the glucans. Molecular mass analysis was performed as described previously, using high performance size exclusion chromatography (HPSEC) coupled on-line with a multi angle laser light scattering (MALLS) and differential refractive index detection (Kralj *et al.*, 2002).

RESULTS

Purification of (mutant) GTFA enzymes

Lb. reuteri 121 GTFA-CHis enzyme expressed in *E. coli* was purified to homogenity. Also three site-directed (D1024N, E1061Q and D1133N; Fig. 1), and eight deletion mutants (GTFA-ΔN-CHis, GTFA-ΔN-NHis, GTFA-ΔNΔ4YG-NHis, GTFA-ΔNΔ5YG-NHis, GTFA-ΔNΔ6YG-NHis, GTFA-ΔNΔ7YG-NHis, GTFA-ΔNΔ8YG-NHis, and GTFA-ΔNΔ11YG-NHis; Fig. 2B) were expressed in *E. coli* and purified to homogenity. The predicted M_r of the different deletion variants (Fig. 2B) was in agreement with the results obtained by SDS-PAGE analysis (data not shown).

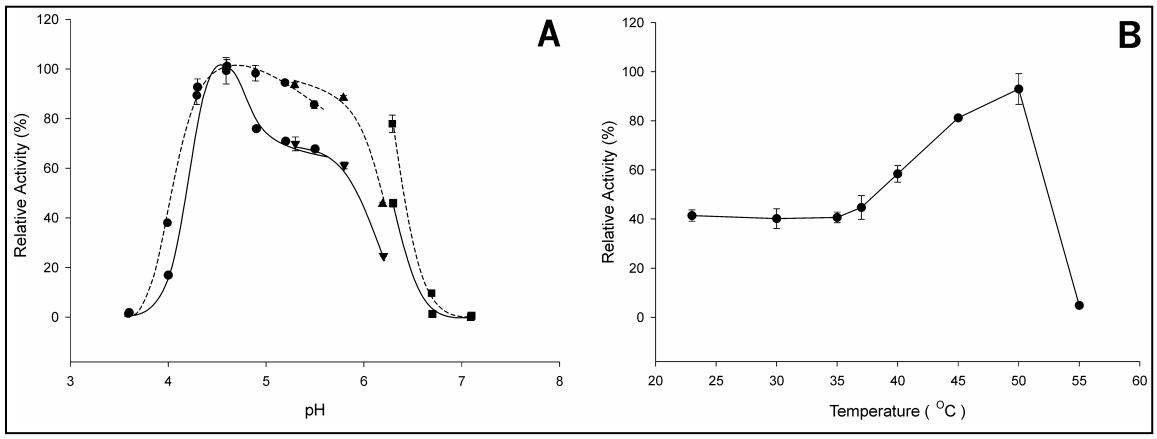

Figure 3. The effect of pH on GTFA activity (**A**). Enzyme activity was determined at 50 °C in the presence of 1 mM $CaCl_2$ by measuring the amount of glucose and fructose released in 30 min from 50 mM sucrose by 30 nM GTFA-CHis (means ± S.E.M.; n = 3). Solid line: Transferase activity. Dashed line: Hydrolysis activity. (●) 25 mM Potassium acetate buffer; (▼) 25 mM MES buffer; (■) 25 mM MOPS buffer. The effect of temperature (**B**) on transferase activity of GTFA was evaluated as described for Figure 3A (means ± S.E.M.; n = 3).

Effects of pH, temperature and metal ions on GTFA activity

In order to define the best conditions for subsequent kinetic studies, the pH and temperature optima of GTFA activity were examined. The pH optimum for the

hydrolyzing activity was in the range of pH 4.5 to 5.5 (Fig. 3A). For the transferase activity a pH optimum at pH 4.7 was observed. The temperature optimum for both reactions was 50 °C. At lower temperatures activity remained relatively high. (Fig. 3B, only shown for transferase activity).

Different metal ions had strongly varying effects on the hydrolytic and transferase activities of GTFA (Table 2). The Zn^{2+}, Hg^{2+}, Fe^{3+}, Fe^{2+} and Cu^{2+} ions significantly inhibited both enzyme activities, but especially the transferase activity, which was not detectable in their presence. EDTA also inhibited transferase activity completely, but hydrolysis remained almost unaffected. In contrast, Ca^{2+}, K^{+}, Na^{2+} and Mg^{2+} ions had stimulating effects on GTFA enzyme activities. Of these compounds Ca^{2+} ions had the most stimulating effect, with hydrolysis and transferase activities increased 2 and 8-fold, respectively.

Compound (1mM)	Hydrolytic activity (%)	Transferase activity (%)
None	100 ±10	100 ± 3
EDTA	89 ± 7	ND
$Cacl_2$	217 ±18	846 ±64
$MgCl_2$	109 ± 5	151 ±13
$Zncl_2$	54 ± 6	ND
KCl	122 ± 7	158 ± 3
HgCl	29 ± 2	ND
$FeCl_3$	26 ± 1	ND
$FeCl_2$	45 ± 1	ND
$CuCl_2$	23 ± 4	ND
NaCl	104 ± 3	121 ± 4

Table 2. Effects of various compounds on GTFA-CHis catalyzed activities. Activity measurements were done at 50 °C in a 25 mM NaAc buffer (pH 4.7), with 50 mM sucrose. Glucose release (V_G, hydrolysis) and fructose minus glucose release (V_F-V_G, transferase) from sucrose by 30 nM GTFA-CHis after 30 min of incubation was taken as a measure for enzymatic activity. Results are given as means ± S.E.M.; n = 3. ND, Transferase activity could not be detected

Kinetic studies of GTFA

(i) Kinetic parameters. In the presence of sucrose GTFA-CHis displayed Michaelis-Menten type of kinetics for the transferase reaction (V_F-V_G) and for total enzyme activity (V_F). The hydrolysis reaction (V_G) displayed Michaelis-Menten with substrate inhibition (Fig. 4A; Table 3). The initial hydrolysis rate represents 95 % of the initial sucrose consumption rate in the presence of 0.5 mM sucrose, 75 % with 2.5 mM sucrose and only 65 % with 10 mM sucrose. These data reveal that GTFA-CHis favours hydrolysis at low sucrose concentrations and polymerization at high sucrose concentrations.

(ii) Effect of maltose on initial GTFA activity. Maltose stimulated the GTFA-CHis total activity (V_F) and transferase activity (V_F-V_G) 3.5 and 7.5 fold, respectively. The hydrolysis (V_G) rate decreased 3.5 fold when maltose was present (Table 3B, data not shown).

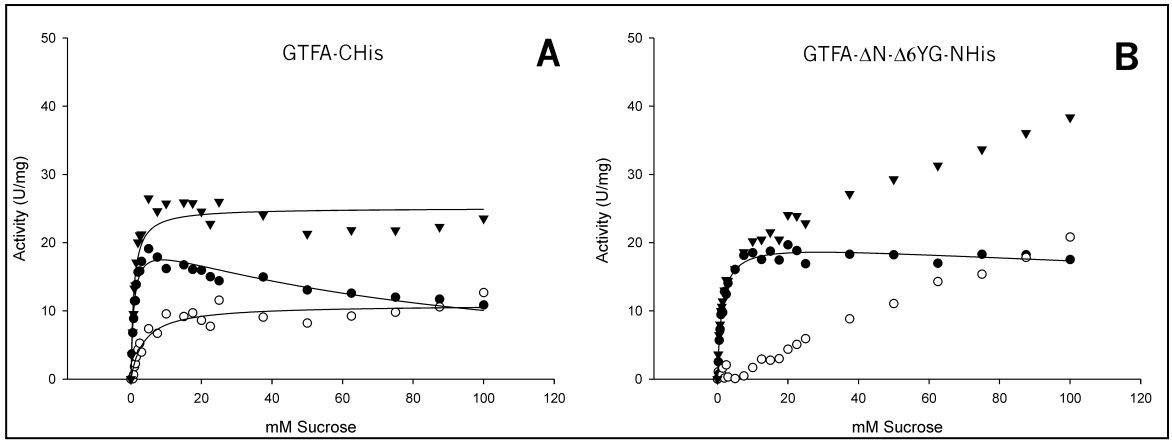

Figure 4. Effect of sucrose concentration on initial GTFA-CHis enzyme activity at 50 °C in 25 mM NaAc buffer pH 4.7 supplemented with 1 mM $CaCl_2$ (**A**). Effect of sucrose concentration on initial GTFA-ΔNΔ6YG-NHis enzyme activity (**B**). The mutants GTFA-ΔNΔ4YG-NHis and GTFA-ΔNΔ5YG-NHis also displayed the latter type of kinetics. The reactions were performed with 30 nM enzyme. (●) V_G (hydrolytic activity), (○) V_G-V_F (transferase activity), (▼) V_F (total activity).

Product analysis

(i) Product spectrum from sucrose. After complete depletion of sucrose, GTFA-CHis showed the following product distribution: reuteran synthesis, 74.8 %; hydrolysis, 23.1 %; and leucrose (α-D-glucopyranosyl-(1→5)-β-D-fructofuranoside) synthesis, 2.1 % (Table 4). Dionex analysis showed that a minor amount of isomaltose and significant amounts of an unknown product that eluted after 35 min were synthesized (data not shown).

(ii) Oligosaccharide synthesis with maltose and isomaltose as acceptor substrates. Dionex analysis showed that, in the presence of maltose, GTFA-CHis formed panose (α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose) as most abundant acceptor reaction product (from 100 mM sucrose and 100 mM maltose, approximately 44 mM panose was synthesized), indicating that an α-(1→6) linkage was formed at the non-reducing end of maltose (Fig. 5B). When isomaltose was used as acceptor low amounts of isomaltotriose and isomaltotetraose (not shown in standard; elution between 35-36 min) were formed, together with two abundant unknown oligosaccharide products (Fig. 5C) with a degree of polymerization (DP) of 3 (most likely isopanose; α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6]-D-glucose) and 4, respectively (DP determined by HPLC, data not shown).

Characteristics of mutants in the putative catalytic amino acid residues

Based on alignments of GTFA with different GTF enzymes, three (putative) catalytic residues were identified in GTFA and mutated (Fig. 1). In the *E. coli* host strain, GTFA mutants D1024N, E1061Q and D1133N were expressed to similar levels as the wild type enzyme (data not shown). Analysis of the purified proteins showed that they had almost completely lost their activity. Compared to wild type, D1024N and E1061Q showed a 1,000 fold reduction of total enzyme activity (V_F). Mutation D1133N resulted in an enzyme with a 300 fold reduced total activity (V_F).

Table 3. Kinetic parameters of wild type and deletion mutants of *Lb. reuteri* 121 GTFA: (**A**) kinetic parameters were determined using 24 different concentrations of sucrose (0.25-100 mM), (**B**) initial total activity determined using 50 mM sucrose and 100 mM maltose. Kinetic parameters for GTFA-ΔN-Δ7YG-NHis, GTFA-ΔN-Δ8YG-NHis and GTFA-ΔN-Δ11YG-NHis were not determined due to their low activity (total activity 43 ×, 63 × and 1000 × reduced, respectively, compared to wild type).*, Affinity for sucrose could not be determined when measuring transferase and total activity of GTFA-ΔN-Δ4YG-NHis, GTFA-ΔN-Δ5YG-NHis and GTFA-ΔN-Δ6YG-NHis, due to failure to reach saturation of the enzyme at the sucrose concentrations used, resulting in high standard errors with curve fits (see also Fig. 4B). Therefore activities at a substrate concentration of 50 mM sucrose are depicted. Data obtained were fitted using the Michaelis-Menten equation (with or without (#) substrate inhibition).

A	***k*cat (s^{-1})**			***K*m (mM)**			***K*i (mM)**	**B**	***k*cat (s^{-1})**
Enzyme	**Total activity**	**Transferase activity**	**Hydrolysis activity**	**Total activity**	**Transferase activity**	**Hydrolysis activity**	**Hydrolysis activity**		**Total activity**
GTFA-CHis	83.1 ± 1.3	36.8 ± 1.0	66.2 ± 1.6	0.9 ± 0.1	4.6 ± 0.6	0.8 ± 0.1	111 ± 12		293
GTFA-ΔN-CHis	188.0 ± 2.2	155.5 ± 2.2	34.2 ± 0.6	2.8 ± 0.1	3.6 ± 0.2	1.0 ± 0.1	-#		354
GTFA-ΔN-NHis	152.0 ± 2.4	112.9 ± 3.7	45.6 ± 0.6	7.9 ± 1.0	3.9 ± 0.3	0.9 ± 0.1	-#		338
GTFA-ΔN-Δ4YG-NHis	139*	67*	81.0 ± 1.8	-*	-*	1.7 ± 0.1	297 ± 48		630
GTFA-ΔN-Δ5YG-NHis	72*	33*	45.3 ± 1.9	-*	-*	1.3 ± 0.2	298 ± 83		429
GTFA-ΔN-Δ6YG-NHis	49*	18*	33.5 ± 0.8	-*	-*	1.3 ± 0.1	635 ± 195		363

Characteristics of GTFA deletion mutants

(i) Glucan binding. GTFA-ΔN-CHis displayed lower glucan binding activity (GBA) than GTFA-ΔN-NHis (Figs. 2B and 6). GTFA-CHis showed virtually no GBA. GTFA-ΔNΔ4YG-NHis completely lacked GBA. GTFA-ΔNΔ5YG-NHis, GTFA-ΔNΔ6YG-NHis and GTFA-ΔNΔ7YG-NHis were active again but showed gradually lower GBA (Fig. 6). GTFA-ΔNΔ8YG-NHis and GTFA-ΔNΔ11YG-NHis were virtually inactive.

(ii) Kinetic analysis. GTFA-ΔN-NHis and GTFA-ΔN-CHis (Fig. 2B) displayed Michaelis-Menten type of kinetics in all three reactions. Interestingly, both mutants had a 3-4 fold increased transferase activity, whereas their hydrolytic activities decreased (Table 3). The Michaelis-Menten formula with a substrate inhibition constant was used to fit the

hydrolytic activity of the mutants GTFA-ΔN-Δ4YG-NHis, GTFA-ΔN-Δ5YG-NHis and GTFA-ΔN-Δ6YG-NHis. The transferase and total activity of these mutants could not be fitted with the Michaelis-Menten formula. The affinity for sucrose had decreased drastically in both reactions and no saturation was reached with the sucrose concentrations used (Fig. 4B). Sequential deletions of the YG-repeats from the C-terminus onwards resulted in initially lower hydrolytic activity compared to the wild type enzyme (Table 3). However, the affinity for sucrose in the hydrolysis reaction was comparable to wild type (Table 3). Kinetic parameters for mutants GTFA-ΔN-Δ7YG-NHis, GTFA-ΔN-Δ8YG-NHis and GTFA-ΔN-Δ11YG-NHis were not determined due to their low total activity (43 ×, 63 × and 1000 × reduced, respectively, compared to wild type levels).

Table 4. Effects of deletions in *Lb. reuteri* 121 GTFA on the product spectrum obtained with sucrose (100 mM), or sucrose and maltose (100 mM each), or sucrose and isomaltose (100 mM each). *Sucrose was not completely consumed. †Oligosaccharide yield is expressed as percentage of total amount of acceptor used in incubation.

Enzyme	Reuteran synthesis (%)	Leucrose synthesis (%)	Hydrolysis (%)	Acceptor reaction oligosaccharide yield† (%) in the presence of	
				Maltose	Isomaltose
GTFA-CHis	74.8 ± 3.5	2.1 ± 0.15	23.1 ± 3.3	59.6 ± 3.9	20.3 ± 1.1
GTFA-ΔN-CHis	79.7 ± 1.8	1.9 ± 0.03	18.4 ± 1.8	57.9 ± 3.1	16.2 ± 0.2
GTFA-ΔN-NHis	82.4 ± 1.3	1.7 ± 0.03	15.8 ± 1.3	55.9 ± 4.4	17.1 ± 0.1
GTFA-ΔN-Δ4YG-NHis	86.0 ± 2.5	1.8 ± 0.08	12.2 ± 2.5	59.1 ± 9.1	22.1 ± 2.6
GTFA-ΔN-Δ5YG-NHis	87.2 ± 2.3	1.8 ± 0.09	11.0 ± 2.2	59.5 ± 5.2	25.0 ± 2.5
GTFA-ΔN-Δ6YG-NHis	85.7 ± 0.6*	1.3 ± 0.26*	13.0 ± 0.4*	57.6 ± 4.6	24.8 ± 2.5

(iii) Effect of maltose on initial mutant GTFA activity. The maltose stimulatory effect on GTFA-ΔN-NHis and GTFA-ΔN-CHis total activity was 1.5 to 2 times lower than on wild type. Total activity of mutants GTFA-ΔN-Δ4YG-NHis, GTFA-ΔN-Δ5YG-NHis and GTFA-ΔN-Δ6YG-NHis on the contrary was stimulated 5.5, 6 and, 7.5 times, respectively, by maltose (Table 3B).

(iv) Product spectrum from sucrose. When incubated with sucrose, mutants with C-terminal deletions showed slightly increased reuteran synthesis and (slightly) decreased hydrolysis (Table 4).

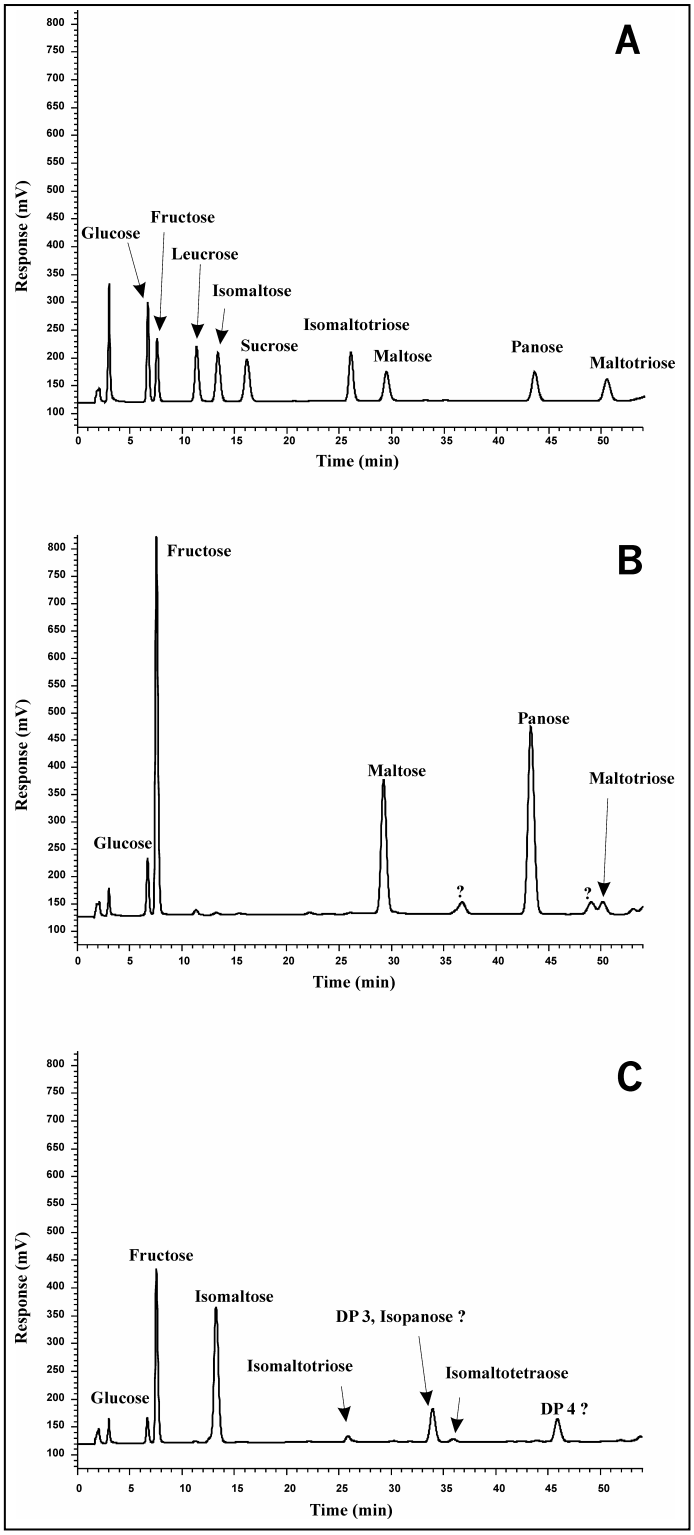

Figure 5. Dionex analysis of *Lb. reuteri* 121 GTFA acceptor reaction products (as described in materials and methods). **A**) Elution profile of a standard mixture, **B**) Products formed upon incubation of 30 nM GTFA-CHis enzyme with 100 mM sucrose and 100 mM maltose for 60 h, **C**) Products formed upon incubation of 30 nM GTFA-CHis enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h.

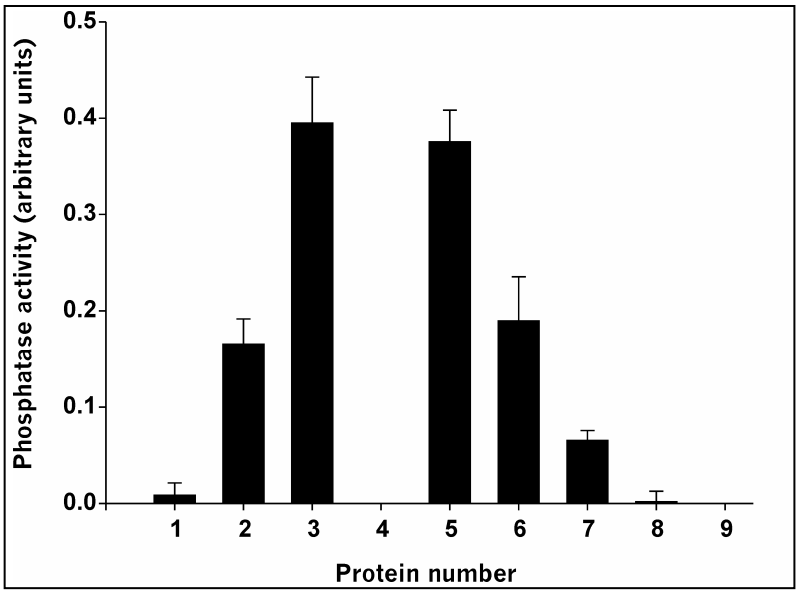

Figure 6. Glucan binding activity of the different *Lb. reuteri* 121 GTFA deletion mutants, measured after 5.5 h of addition of substrate (means ± S.E.M.; n = 3). Protein numbers refer to the various (deletion) mutants shown in Fig. 2B.

(v) Oligosaccharide synthesis with maltose and isomaltose as acceptor substrates. In the acceptor reaction, all deletion mutants showed similar oligosaccharide yields as wild type (Table 4). The deletion mutants produced the same oligosaccharides as wild type (Fig. 5, data not shown).

Table 5. Methylation analysis and molecular masses of the glucans produced by purified wild type *Lb. reuteri* 121 GTFA enzyme and derived deletion mutants.

Type of glucosyl unit	**Methylation (%)**					
	GTFA-CHis	**GTFA-ΔN-CHis**	**GTFA-ΔN-NHis**	**GTFA-ΔN-Δ4YG-NHis**	**GTFA-ΔN-Δ5YG-NHis**	**GTFA-ΔN-Δ6YG-NHis**
Terminal	9	9	8	9	5	5
α-(1→4)	49	46	49	48	46	47
α-(1→6)	26	34	28	29	40	40
α-(1→4,6)	15	12	15	14	9	8
	Molecular mass (1×10^6 Da)					
	45	50	48	47	38	39

(vi) Molecular sizes and linkage type analysis of glucans produced. Compared to GTFA-CHis, the deletion mutants showed no drastic changes in the sizes and linkage types of the glucans produced (Table 5). However, the glucan products of mutants GTFA-ΔNΔ5YG-NHis and GTFA-ΔNΔ6YG-NHis showed less branching (lower percentage of

both terminal and branched, α-(1→4,6) glucosyl units), a higher percentage of α-(1→6) linkages, and slightly decreased molecular masses compared to wild type enzyme and other deletion mutants (Table 5).

DISCUSSION

Characterization of GTFA

This paper reports a detailed biochemical and molecular characterization of the first recombinant glucansucrase (GTFA) of a *Lactobacillus* strain, synthesizing a polymer with mainly α-(1→4) glucosidic linkages (a reuteransucrase).

The GTFA pH optimum was comparable to that of other glucansucrases. Interestingly, transferase activity showed a sharp optimum around pH 4.7 whereas the hydrolysis reaction had a broader optimal pH range (Fig. 3A). This is the first time demonstration of different pH profiles for these GTF catalyzed reactions. A striking feature of the recombinant GTFA-CHis protein is its high optimal temperature of 50 °C (Fig. 3B). This high temperature optimum was also observed for the inulosucrase and levansucrase enzymes of the same strain (van Hijum *et al.*, 2002, van Hijum *et al.*, 2003, van Hijum *et al.*, 2004).

Of all cat-ions tested Ca^{2+} had the most stimulating effect on enzyme activity (Table 2): hydrolysis and transferase activity were increased 2 and 8-fold, respectively. The Zn^{2+}, Hg^{2+}, Fe^{3+}, Fe^{2+} and Cu^{2+} ions significantly inhibited enzyme activity. These compounds (except for Hg^{2+} which was not tested) also inhibited the *gtfB*, *gtfC* and *gtfD* encoded enzymes of *Streptococcus mutans* (Wunder & Bowen, 1999).

Kinetics of GTFA

Kinetic analysis of GTFA-CHis revealed interesting features (Fig. 4). At high sucrose concentrations, substrate inhibition was observed for the GTFA-CHis hydrolysis reaction. GTFA-CHis clearly favours hydrolysis at low sucrose concentrations and transferase at high sucrose concentrations. The same phenomenon has otherwise been observed only for the family 13 enzyme amylosucrase from *Neisseria polysaccharea* (Potocki de Montalk *et al.*, 2000).

The GTFA affinity for sucrose in the total reaction was much higher (*K*m of 0.9 mM) than observed for GTFI of *S. downei* Mfe28 and DSRS of *Ln. mesenteroides* NRRL B-512F (*K*m values of 49 and 26 mM, respectively) (Monchois *et al.*, 2000a, Monchois *et al.*, 1997). The stimulating effect of maltose on initial GTFA velocity (3-4 fold activation of total activity) was also observed for DSRS of *Ln. mesenteroides* NRRL B-512F and GTFI of *S. downei* Mfe28 (2- and 4 fold stimulation of total activity, respectively) (Monchois *et al.*, 2000a, Monchois *et al.*, 1997).

Product spectrum from sucrose

Upon depletion of sucrose, GTFA had formed only low levels of leucrose (2.1 % of total sucrose consumed), even when incubated together with a high concentration of fructose 100 mM (about 5 % leucrose synthesized) (Table 4; data not shown). GTFI of *S. downei* on the contrary produced significant amounts of leucrose (24.0 %) (Monchois *et al.*, 2000b, Monchois *et al.*, 2000a). GTFA synthesized more glucan (74.8 %), and was more effective in hydrolysis (23.1 %) than GTFI of *S. downei* Mfe28 (60.0 % and 16.2 %, respectively) (Monchois *et al.*, 2000a). Fructose thus is a better acceptor for GTFI of *S. downei* Mfe28 than for GTFA of *Lb. reuteri* 121.

Oligosaccharide synthesis with maltose and isomaltose as acceptors

Analysis of the acceptor reaction of GTFA revealed that with glucose and sucrose only a small amount of isomaltose was formed (data not shown). Previous studies showed that the production of isomaltose by DSRS from *Ln. mesenteroides* NRRL B-512F could be improved by using high amounts of sucrose and glucose (Buchholz & Seibel, 2003).

Maltose is a good acceptor reaction substrate for DSRS of *Ln. mesenteroides* B-512F (Robyt & Walseth, 1978, Monchois *et al.*, 1997), for GTFI of *S. downei* Mfe28 (Monchois *et al.*, 1999c), and for GTFA of *Lb. reuteri* 121 (this study). GTFA produced panose as major product with maltose as acceptor (formation of an α-(1→6) linkage to the non-reducing end of maltose), together with low amounts of maltotriose and two unknown products (Fig. 5B). The formation of panose has also been observed for other GTF enzymes (Monchois *et al.*, 1997, Monchois *et al.*, 1998a, Monchois *et al.*, 1996, Monchois *et al.*, 2000b, Robyt, 1996). Compared to GTFI of *S. downei* Mfe28 (91.3 %) and DSRS of *Ln. mesenteroides* NRRL-B512F (93 %) (Monchois *et al.*, 2000a, Monchois *et al.*, 1998b), the yield of oligosaccharides synthesized by GTFA-CHis with maltose as acceptor substrate (59.6 %) is lower. Maltose thus is a better acceptor reaction substrate for GTFI and DSRS than for GTFA.

When isomaltose was used as acceptor reaction substrate, two unknown products (identified by HPLC as DP 3 and DP 4, data not shown) were produced. Maltotriose or panose eluted at different time points than the DP 3 oligosaccharide; its most likely identity is isopanose. The coupling of a glucose moiety, using one of the two linkage types synthesized by GTFA, α-(1→4) and α-(1→6), to the reducing end of isomaltose would have resulted in the formation of isomaltotriose (of which small amounts are indeed synthesized), or panose (not formed) (Fig. 5C). However, various studies show that glucansucrases elongate oligosaccharides at their non-reducing end (Mukasa *et al.*, 2000, Dols *et al.*, 1997, Arguello Morales *et al.*, 2001, Monchois *et al.*, 2000a). The small amount of isomaltotriose is thus probably formed by coupling of glucose with an α-(1→6) linkage to the non-reducing end of isomaltose. Linkage specificity (inside glucans) of several glucansucrases is conserved in oligosaccharide synthesis (Dols *et al.*, 1997, Cote

& Robyt, 1982, Robyt & Walseth, 1978). This suggests that the DP 3 oligosaccharide is isopanose (coupling of a glucose unit by an α-(1→4) glucosidic bond onto the non-reducing end of isomaltose). Besides the DP 3 oligosaccharide, also a DP 4 oligosaccharide was synthesized. The exact nature of both oligosaccharides will be analyzed in future. However, it appears likely that reuteransucrase is capable of forming both α-(1→4) and α-(1→6) glucosidic bonds with acceptor reaction substrates (Fig. 5).

Characteristics of catalytic mutants

Mutagenesis of the *Lb. reuteri* strain 121 GTFA putative catalytic residues (mutants D1024N, E1061Q and D1133N) resulted in drastically reduced total enzymatic activities. D1024 of GTFA is homologous to: 1) the Asp residues identified by Mooser et al. (Mooser *et al.*, 1991) as part of the active site of Streptococcus sobrinus GTFI and GTFS, respectively, 2) Asp451 of GTFB from *S. mutans* GS5, 3) Asp453 of GTFI from S. downei Mfe28 and 4) Asp551 of DSRS of *Ln. mesenteroides* NRRL-B512F, which were also shown to be essential for activity (Kato *et al.*, 1992, Monchois *et al.*, 1997, Devulapalle *et al.*, 1997) (Fig. 1). GTFA residues E1061 and D1133 correspond to residues E491 and D564 in GTFI of *S. downei* Mfe28. These residues are essential for activity in both GTFI and GTFA (Devulapalle *et al.*, 1997); this study)

Characteristics of deletion mutants

GTFA possesses a relatively large N-terminal variable domain (702 amino acids) containing 5 RDV repeats (Kralj *et al.*, 2002). Its complete deletion had a drastic effect on enzyme kinetics. The initial transferase activity of GTFA-ΔN-CHis and GTFA-ΔN-NHis increased 3-4 fold, whereas the hydrolytic activity decreased (Table 3). Conceivably, the large N-terminal domain in the wild type protein causes steric hindrance to the growing glucan chain, its deletion resulting in a strongly increased transferase activity. The affinities for the substrate sucrose remained rather similar, in both the transferase and the hydrolysis reaction (Table 3). The data show that the large N-terminal variable domain is important for initial activity with sucrose (Table 3), but has only small effects on the product spectrum with sucrose alone, or sucrose plus acceptor substrates (Table 4), and on glucan characteristics (Table 5).

The GTFA mutants with N-terminal and additional C-terminal deletions showed drastically decreased affinity for sucrose in the total (V_F) and transferase (V_F-V_G) reactions (Fig. 4B; Table 3). In contrast, C-terminal deletions in DSRS of *Ln. mesenteroides* NRRL B-512F had virtually no effects on *K*m values with sucrose (Monchois *et al.*, 1998b). Sequential deletions of the YG-repeats from the C-terminus onwards resulted in gradually lower activity of the mutant GTFA enzymes in all reactions (Table 3A). The stimulatory effect of maltose increased upon deletion of C-terminal YG-repeats (Table 3B). Both phenomena were also observed for DSRS from *Ln. mesenteroides* NRRL B-512F

(Monchois *et al.*, 1998b). The increased stimulatory effect by maltose upon C-terminal deletions in DSRS was explained by a change in a rate-limiting reaction step. C-terminal deletions in GTFI from *S. downei* Mfe28 had no effect on distribution of glucosyl residues during mutan synthesis (Monchois *et al.*, 1999a). The reuterans produced by wild type GTFA and C-terminal deletion mutants derived had comparable sizes and linkage type distribution, except for GTFA-ΔNΔ5YG-NHis and GTFA-ΔNΔ6YG-NHis. The latter mutants showed less branching (lower amount of both terminal and branched, α-(1→4,6) glucosyl units), a higher percentage of α-(1→6) linkages and a slightly decreased molecular mass compared to the wild type and other mutants (Table 5). C-terminal deletions in GTFI of *S. downei* Mfe28 did not affect glucan structure (Monchois *et al.*, 1999a). However, deletion of six internal units (A-C) of GTFG of *Streptococcus gordonii* also affected glucan structure (Vickerman *et al.*, 1996). GTFA enzyme activity could still be detected after removal of more than half (152 amino acids, 6-YG-repeats) of the GBD (Table 3). When more than 6-YG-repeats were removed enzyme activity was reduced drastically (Table 3). C-terminal deletions in other glucansucrases also affected enzyme activity (Monchois *et al.*, 1998b, Kato & Kuramitsu, 1990). Only in case of GTFI of *S. downei* Mfe28 the C-terminal domain could be removed completely without large loss of activity (Monchois *et al.*, 1999a).

The C-terminal domains of glucansucrases from leuconostocs and streptococci are involved in glucan binding (Abo *et al.*, 1991, Shah & Russell, 2002, Lis *et al.*, 1995, Monchois *et al.*, 1998b). The A repeats found in the C-terminal domains of these GTF enzymes are not present *Lb. reuteri* 121 GTFA. Instead, 11 YG-repeats were identified (Kralj *et al.*, 2002). Their deletion revealed that the GTFA C-terminal domain and these YG-repeats are involved in glucan binding (Fig. 6). GTFA-ΔNΔ4YG-NHis was not able to bind biotin-dextran, which may be due to conformational changes in this mutant protein. GTFA-CHis showed virtually no GBA. The location of the His-tag (GTFA-ΔN-CHis also showed lower GBA than GTFA-ΔN-NHis), together with the presence of the large N-terminal variable domain, may block the access of the GBD to the biotin-dextran. We had to incubate the GTFA protein / biotin-dextran / extravidin-alkaline phosphatase complex for several hours with the 4-nitrophenyl phosphate substrate to be able to measure GBA, whereas in a previous report, with GFTI of *S. downei* Mfe28, incubation for a few minutes was enough to measure comparable amounts of GBA (Shah & Russell, 2002). Reuteran rather than biotin-dextran may be the preferred ligand for the C-terminal repeats of GTFA. Nevertheless, we show for the first time that also in reuteransucrase, an α-(1→4) synthesizing glucansucrase from a *Lactobacillus* strain, the C-terminal domain is involved in glucan binding. Furthermore, positions at which truncation prevented glucan binding (deletion of 8 YG-repeats; 100 amino acids after the end of catalytic core) were identified (Fig. 6).

The GTFA hydrolytic activity remained upon deletion of YG-repeats, whereas glucan binding and affinity for sucrose in the transferase reaction decreased. This may imply that the glucan binding domain is involved in polymer chain growth, as previously postulated for the GTFI from *S. mutans* GS5 and GTFI from *S. sobrinus* 6715 (Kato & Kuramitsu, 1990, Abo *et al.*, 1991).

Conclusion

A thorough molecular and biochemical investigation of GTFA, the first GTF enzyme synthesizing linkages of the α-(1→4) glucosidic type, has been carried out. This includes analysis of the kinetics of wild type enzyme and N- and C-terminally truncated mutants derived, of the acceptor reaction, and of glucan binding by the C-terminal domain. Finally three catalytically important residues have been identified and characterized.

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Chapter 4

A highly hydrolytic reuteransucrase from a probiotic *Lactobacillus reuteri* strain

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SUMMARY

Lactobacillus reuteri strain "BioGaia" was isolated as a pure culture from a Reuteri™ Tablet purchased from the BioGaia company. We found that this probiotic strain produces a soluble glucan (reuteran), in which the majority of the linkages are of the α-(1→4) glucosidic type (~70%). This reuteran also contains α-(1→6) linked glucosyl units and 4,6-disubstituted α-glucosyl units at the branching points. The *Lb. reuteri* BioGaia glucansucrase gene (*gtfBio*) was cloned, expressed in *Escherichia coli* and the GTFBIO enzyme was purified. The recombinant GTFBIO enzyme and the *Lb. reuteri* strain BioGaia culture supernatants synthesized identical glucan polymers with respect to binding type and size distribution. GTFBIO is thus responsible for synthesis of this reuteran polymer in *Lb. reuteri* strain BioGaia. The preference of GTFBIO for synthesizing α-(1→4) linkages is also evident from the oligosaccharides produced from sucrose with different acceptor substrates, e.g. isopanose from isomaltose. GTFBIO has a relatively high hydrolysis/transferase activity ratio. Complete conversion of 100 mM sucrose by GTFBIO nevertheless yielded large amounts of reuteran, although more than 50 % of sucrose was converted into glucose. This is only the second example of the isolation and characterization of a reuteransucrase and its reuteran product, both found in different *Lb. reuteri* strains. GTFBIO synthesizes a reuteran with the highest amount of α-(1→4) linkages reported to date.

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive microorganisms. The core of LAB are historically formed by the genera: *Pediococcus*, *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Lactobacillus* (Axelsson, 1998). Members of these genera are used for several food (and feed) applications (e.g. silage, dairy products, vegetables, sourdough, fish and meats) (Lindgren & Dobrogosz, 1990, Tieking *et al.*, 2003). Many lactic acid bacteria produce an abundant variety of exopolysaccharides (EPS), heteropolysaccharides and homopolysaccharides (de Vuyst & Degeest, 1999, Monchois *et al.*, 1999d), which may be used to develop a new generation of food-grade ingredients.

α-Glucans are an example of homopolysaccharides synthesized from sucrose by large extracellular enzymes, glucosyltransferases (EC 2.4.1.5, commonly named glucansucrases, GTFs). Glucansucrase enzymes catalyze two different reactions, depending on the nature of the acceptor: i) hydrolysis, when water is used as acceptor; ii) glucosyl transfer (transferase), which can be divided in: a) polymerization, when the growing glucan chain is used as acceptor, and b) oligosaccharide synthesis, when oligosaccharides (e.g. maltose, isomaltose) are used as acceptor. Where studied, the linkage specificity of glucansucrases

is conserved in oligosaccharide synthesis (Dols *et al.*, 1997, Cote & Robyt, 1982, Robyt & Walseth, 1978), and oligosaccharides are elongated at their non-reducing end (Dols *et al.*, 1997, Arguello Morales *et al.*, 2001, Monchois *et al.*, 2000a, Mukasa *et al.*, 2000).

Glucansucrase enzymes have been found in *Leuconostoc*, *Streptococcus* and *Lactobacillus* sp., (Monchois *et al.*, 1999d, van Geel-Schutten *et al.*, 1999, Tieking *et al.*, 2003, Kralj *et al.*, 2003, Kralj *et al.*, 2004b) synthesizing glucans with α-(1→2), α-(1→3), α-(1→4) or α-(1→6) glucosidic linkages. Only a single glucansucrase enzyme synthesizing a glucan with α-(1→4) and α-(1→6) glucosidic bonds recently has been characterized, namely GTFA of *Lactobacillus reuteri* 121 (Kralj *et al.*, 2002, Kralj *et al.*, 2004d).

Glucansucrase from *Lactobacillus* (*reuteri*) strains are of special interest for different reasons: (i) Some *Lb. reuteri* strains have probiotic properties as has been demonstrated in various animals and humans (Casas *et al.*, 1998, Valeur *et al.*, 2004). (ii) The range of glucans and oligosaccharides produced by GTF enzymes present in lactobacilli (Kralj *et al.*, 2004d) may potentially act as prebiotic by stimulating the growth of probiotic strains or of beneficial endogenic strains of the gastrointestinal tract (Monsan & Paul, 1995, Olano-Martin *et al.*, 2000, Chung & Day, 2002). (iii) Several *Lb. reuteri* strains are able to produce anti-microbial metabolites (e.g. reutericyclin, reuterin and reutericin), which delay growth of some food borne pathogens (Kabuki *et al.*, 1997, Ganzle *et al.*, 2000, Talarico *et al.*, 1988). (iv) GTF enzymes of *Leuconostoc* require specific induction by sucrose, which is disadvantageous for several applications (Kim & Robyt, 1994). The GTF enzymes of *Streptococcus* sp. are generally produced constitutively (Kim & Robyt, 1994). Also reuteransucrase from *Lb. reuteri* 121 (van Geel-Schutten *et al.*, 1999) and dextransucrases from *Lb. reuteri* 180 and *Lactobacillus parabuchneri* 33 (Kralj *et al.*, 2003, Kralj *et al.*, 2004b) are produced constitutively (unpublished data). (v) The only streptococcal species that is associated with food technology is *Streptococcus thermophilus*, which is used in the manufacture of yoghurt. The genus *Streptococcus* contains several well-known pathogens (e.g. *Streptococcus pneumoniae*) (Axelsson, 1998, Leroy & de Vuyst, 2004). Furthermore, glucans produced by oral streptococci play a key role in the cariogenesis process, by enhancing the attachment and colonization of cariogenic bacteria (Loesche, 1986). *Leuconostoc* strains play an important role in vegetable fermentations (Axelsson, 1998, Leroy & de Vuyst, 2004). Lactobacilli are widespread in nature and many species have found applications in the food industry (e.g. dairy products, sourdough) (Axelsson, 1998, de Vuyst & Degeest, 1999). Glucans and glucooligosaccharides from lactobacilli are thus interesting and feasible alternatives for the additives currently used in the production of foods (e.g. sourdough, yoghurts, health foods).

Different *Lactobacillus* strains are able to produce glucans (Kralj *et al.*, 2003, Kralj *et al.*, 2004b, Tieking *et al.*, 2003, Sidebotham, 1974, van Geel-Schutten *et al.*, 1999, van

Geel-Schutten *et al.*, 1998). Besides GTFA of *Lb. reuteri* 121 (Kralj *et al.*, 2002), six other *Lactobacillus* (*reuteri*) glucosyltransferase enzymes have been recently characterized (Kralj *et al.*, 2003, Kralj *et al.*, 2004b). They were found to synthesize glucan polymers containing α-(1→3) (mutan) or α-(1→6) (dextran) glucosidic linkages in various ratios (Kralj *et al.*, 2004b). This paper describes the molecular and biochemical characterization of a reuteransucrase gene (*gtfBio*), and the novel reuteransucrase (GTFBIO) encoded from a probiotic *Lb. reuteri* strain used by the BioGaia company (www.biogaia.com). The *gtfBio* gene encodes a novel reuteransucrase (GTFBIO) with a high hydrolysis/transferase activity ratio, which synthesizes a branched glucan (reuteran) containing the highest amount of α-(1→4) glucosidic linkages reported to date.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions

Lb. reuteri strain BioGaia (LB BIO) was isolated as a pure culture from a "Reuteri™ Tablet" (BioGaia AB, Stockholm, Sweden; www.biogaia.com) and cultivated anaerobically at 37 °C in MRS medium (Difco) (De Man *et al.*, 1960) or in MRS-s medium (i.e. MRS-medium with 100 g l^{-1} sucrose instead of 20 g l^{-1} glucose). The taxonomic position of *Lb. reuteri* BioGaia was confirmed by 16s rDNA analysis (99 % identity within 1537 nucleotides with the *Lb. reuteri* type strain DSM 20016 T). *E. coli* TOP 10 (Invitrogen) and plasmid pCR-XL-TOPO (Invitrogen) were used for cloning of the *gtf* gene and for sequencing purposes. Plasmid pBluescript II SK^+ (Stratagene) was used for cloning of the complete *gtf* gene. Plasmid pET15b (Novagen) was used for expression of the *gtf* gene in *E. coli* BL21 Star (DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium (Ausubel *et al.*, 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (100 μg ml^{-1} ampicillin or 50 μg ml^{-1} kanamycin). Agar plates were made by adding 1.5% agar to the LB medium; when appropriate X-gal (20 μg ml^{-1}) was added.

Molecular techniques

Lb. reuteri strain BioGaia total DNA was isolated according to (Nagy *et al.*, 1995). Plasmid DNA of *E. coli* was isolated using the alkaline lysis method (Birnboim & Doly, 1979) or with a Wizard Plus SV plasmid extraction kit (Promega). General procedures for cloning, *E. coli* transformations, DNA manipulations, and agarose gel electrophoresis were as described (Sambrook *et al.*, 1989). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the enzyme suppliers (New England Biolabs; Roche Biochemicals). Primers were obtained from Eurogentec. Sequencing was performed by GATC (Germany). DNA was amplified by PCR on a DNA

Thermal Cycler PTC-200 (MJ Research) using *Pwo* DNA polymerase (Roche Biochemicals) or Expand High Fidelity polymerase (Roche Biochemicals). For inverse PCR (iPCR) the Expand High Fidelity PCR system (Roche Biochemicals) was used as described by the supplier. Fragments were isolated from agarose gels using a Qiagen gel extraction kit (Qiagen) following the instructions of the supplier.

Identification and nucleotide sequence analysis of the *gtf* gene

An overview of the isolation strategy of the *gtf* gene is given in Fig. 1. The first fragment of the *gtf* gene was isolated by PCR amplification of chromosomal DNA of the *Lb. reuteri* strain BioGaia using degenerate primers (DegFor and DegRev) based on sequence homology between conserved regions, located in the catalytic core of different *gtf* genes of Gram-positive bacteria (see (Kralj *et al.*, 2003)). Amplification products with the expected size of about 660 bp were ligated into pCR-XL-TOPO (Invitrogen) and transformed to *E. coli* TOP 10. From ten random clones, plasmid DNA was isolated and analyzed by restriction using *Eco*RI and *Nsi*I. Subsequently five of the ten clones were sequenced (GATC, Germany), revealing identical nucleotide sequences and confirming *gtf* gene identity. The 660 bp amplified fragment was used to design the primers Bio1For 5'- GGAACGATTACCGGCAATAACGACCAGGCG-3' and Bio1Rev 5'- GTTCAGGA GGTAGTAGAGCCAGTTCAGCTG-3'; BioFor2 5'- CATACGCAATCCTCCTAACCA ACAAGGATACG-3' and BioRev2 5'-GGACGGAATATTGTACAGGTTGTACTTCTT AAC-3' for two separate inverse PCR reactions (Fig. 1) (Triglia *et al.*, 1988). *Lb. reuteri* strain BioGaia chromosomal DNA was digested with *Eco*RI and ligated, yielding circular DNA molecules. PCR with diverging primers Bio1For and Bio1Rev // BioFor2 and BioRev2 (Fig. 1), with the circular ligation products as template yielded amplicons of ~6000 bp (5'-sequence) and ~3000 bp (3'-sequence), respectively. After cloning of both fragments into pCR-XL-TOPO (Invitrogen) their nucleotide sequences were determined (GATC, Germany).

Construction of plasmids for expression of the GTFBIO encoding (mutant) genes in *E. coli*

Two separate PCR reactions were used to amplify the complete *gtf* gene, without signal sequence and with a C-terminal His-tag (1750 amino acids). The product of the first PCR reaction, synthesized with primers FBiocomp 5'- GATGCAT**GAGCTC**ATGA ATACCACCACGCCGGCTGATAACCAGTCTG-3', containing *Sac*I (bold) and *Bsp*HI (underlined) sites, and BioPstRev 5'- GTCAAACAGCCGGTCTAATGAAACGGTTCC-3', was digested with *Sac*I/*Pst*I. The product of the second PCR reaction, primed with oligo's BioPstFor 5'- GCAGTACATTCAGTCTTATTCACCAGTATTG-3'and BioRevHis 5'- ATATCGAT**CTCGAG**CGGATCCTATTA*GTGATGGTGATGGTGAT*

*G*AATTCTATTTTGATCAGTCATCTGACCAATATCAG-3', containing *Xho*I (bold), *Bam*HI (underlined) sites and a 6x His-tag (italics) was digested with *Pst*I/*Xho*I. The resulting fragments (~2200 bp) and (~3100 bp) were cloned in the corresponding sites of pBluescript II SK^+, yielding pBSP2200 and pBPX3100. Both plasmids were digested with *Sac*I/*Pst*I and the pBSP2200 fragment containing the 5' part of *gtf* was ligated into pBPX3100 yielding pBGTFBIO. Plasmid pBGTFBIO was digested with *Bsp*HI/*Bam*HI and the resulting 5.3 kb fragment was ligated into the *Nco*I/*Bam*HI sites of the expression vector pET15b (Novagen) yielding p15GTFBIO. To construct an N-terminal deletion variant of GTFBIO (1042 amino acids), devoid of its signal sequence, N-terminal variable region and with a C-terminal His-tag, a PCR reaction was carried out with primers FBioCore 5'-GATGCAT**GAGCTC**ATGAAGGATGGCAAGGATTACTACTATGACC C-3', containing *Sac*I (bold) and *Bsp*HI (underlined) sites, and BioRevHis (see above sequence), was digested with *Bsp*HI/*Bam*HI and the resulting 3.1 kb fragment was ligated into the *Nco*I/*Bam*HI sites of the expression vector pET15b (Novagen) yielding p15GTFBIO-ΔN.

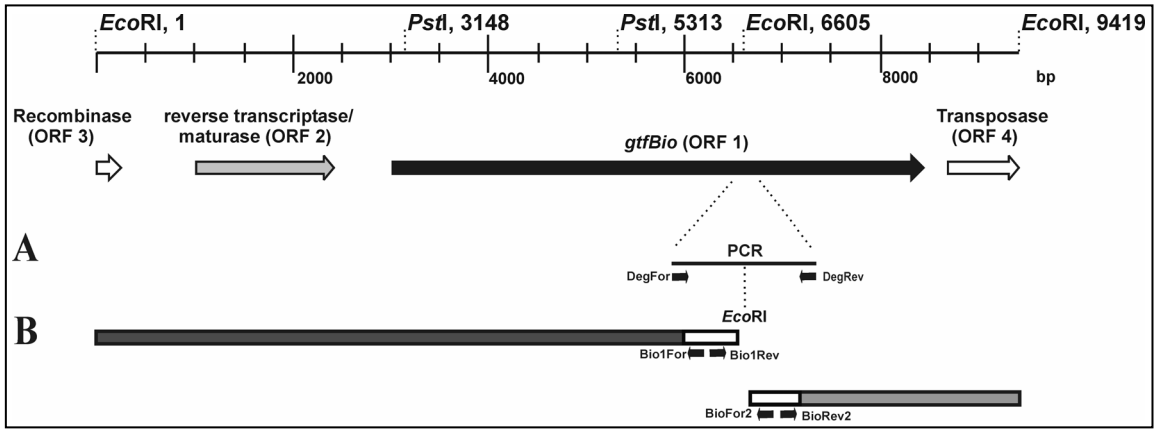

Figure 1. Strategy used for the isolation of the *gtfBio* gene and surrounding regions from *Lb. reuteri* strain BioGaia chromosomal DNA. Primers are indicated with small arrows. The fragment shown in Fig. **1A** is the ~660 bp PCR product isolated with degenerate primers. The fragments in Fig. **1B** are regions amplified by inverse PCR (~6000 bp) and (~3000 bp), respectively. Partial ORFs are shown as open arrows.

Purification of GTFBIO(-ΔN) proteins

Cells of *E. coli* BL21star (DE3) harbouring p15GTFBIO(-ΔN) were harvested by centrifugation (10 min at 4 °C at 10,000 × *g*) after 16 h of growth at 37 °C (without induction). The pellet was washed with 50 mM phosphate buffer pH 8.0. Pelleted cells were resuspended in 50 mM sodium phosphate buffer pH 8.0 containing 250 mM NaCl, 5

mM β-mercaptoethanol and 10 mM imidazole. Cells were broken by sonication (7 × 15 sec at 7 micron with 30 sec intervals) and centrifuged (10 min at 4 °C at 10,000 × *g*). The clear lysate containing GTF activity was loaded on a Ni-NTA column (Qiagen). Binding was achieved using 50 mM sodium phosphate buffer pH 8.0 containing 250 mM NaCl, 5 mM β-mercaptoethanol and 10 mM imidazole, followed by washing using the same buffer. Elution of His-tagged protein(s) was performed using 50 mM sodium phosphate buffer pH 8.0 with 250 mM NaCl, 1 mM β-mercaptoethanol and 200 mM imidazole. Eluted proteins were desalted with 20 mM Tris buffer, pH 8.0, using a 5 ml Hi-Trap desalting column (Amersham Pharmacia Biotech). Subsequently, the samples were purified on an ACTA prime FPLC system (Amersham Pharmacia Biotech), using a 1 ml Resource-Q column (Amersham Pharmacia Biotech) and a linear gradient of 30 ml with 1 M NaCl in 20 mM Tris buffer pH 8.0 as eluens at a flow rate of 1 ml.min^{-1}. Proteins present in the elution peak were desalted with 25 mM sodium acetate buffer pH 5.0, supplemented with 1 mM $CaCl_2$, using a 5 ml Hi-Trap desalting column (Amersham Pharmacia Biotech). At each stage of the purification, the GTF transferase activity was quantified as previously described (van Geel-Schutten *et al.*, 1999). The degree of purity was determined by SDS-PAGE (Laemmli, 1970).

Protein concentrations were determined using the Bradford method using the Bio-Rad reagent and BSA (bovine serum albumin) as a standard (Bio-Rad).

Enzyme activity assays

The various reuteransucrase activities were determined as initial rates by measuring glucose and fructose release (enzymatically) from sucrose conversion (van Geel-Schutten *et al.*, 1999, Kralj *et al.*, 2004d). The amount of fructose released (V_F) corresponds to total enzyme activity (initial formation of leucrose and other sucrose isomers was negligible, see results and data not shown). The amount of free glucose (V_G) represents the hydrolytic activity of the enzyme. The amount of fructose minus the amount of free glucose reflects the transferase activity (V_F-V_G). Unless indicated otherwise, reactions were performed at 35 °C in 25 mM NaAc buffer, pH 5.0, containing 1 mM $CaCl_2$ and 30 nM purified GTFBIO enzyme. One unit of enzyme activity is defined as the release of 1 µmol of monosaccharide per min.

(i) pH and temperature optima. Enzyme activity was determined at 35 °C in the presence of 1 mM $CaCl_2$ by measuring the amount of glucose and fructose released in 30 min from 50 mM sucrose by 30 nM GTFBIO.

(ii) Kinetic parameters. Kinetic assays were performed with fifteen different sucrose concentrations ranging from 0.25 to 100 mM. Over a 6 min incubation period, samples of 25 µl were withdrawn every minute and inactivated with 2.5 µl 1 M NaOH. Curve fitting

of the data was performed with the "SigmaPlot" program (version 8.0) using the Michaelis-Menten formula [$y = (a \times x) / (b + x)$]. In this equation y is the specific activity ($U.mg^{-1}$), x is the substrate concentration (mM sucrose), a is the maximal reaction rate, Vmax ($U.mg^{-1}$), and b is the affinity constant for the substrate (Km, mM sucrose).

(iii) Effect of maltose on initial GTFBIO activity. The initial rate of oligosaccharide synthesis was examined by measuring the effect of maltose on GTFBIO enzyme activity, using 50 mM sucrose and 100 mM maltose. The activity was determined by measuring fructose release.

Product analysis

(i) Product spectrum from sucrose. After complete depletion of sucrose (100 mM, 60 h at 35 °C) by 30 nM GTFBIO enzyme, the concentrations of fructose, glucose, isomaltose and leucrose in the reaction medium of GTFA (mutants) were determined using anion exchange chromatography (see below). The amount of free fructose released from sucrose (97.9 %) and leucrose synthesized from sucrose (2.1 %) corresponds to 100 % sucrose converted. Subtracting the free glucose released from sucrose (22.7 %; due to hydrolysis), plus isomaltose (1.6 %) synthesized from sucrose, from the free fructose (97.9 %) released from sucrose, allowed calculation of the yield of reuteran synthesis (73.7 %) from sucrose (data of GTFA used here for clarification, see Table 1B).

(ii) Oligosaccharide synthesis with maltose and isomaltose as acceptors. Oligosaccharide synthesis was analyzed using 100 mM sucrose together with maltose or isomaltose (100 mM each). After complete consumption of sucrose (60 h at 35 °C), samples were diluted 500-1000 times in a 90% DMSO solution. Maltose, isomaltose, maltotriose, panose (Sigma), isomaltotriose, isomaltotetraose (TNO Nutrition and Food Research, Groningen, The Netherlands), sucrose (Acros Organics), fructose, glucose (Merck), and leucrose (Pfeiffer & Langen) were used as standards. The percentage of oligosaccharide synthesis from sucrose and acceptor was determined by subtracting the amount of unused acceptor from the initial acceptor concentration. Separation of oligosaccharides was achieved with a CarboPac PA1 anion exchange column (250 × 4 mm; Dionex) coupled to a CarboPac1 guard column (Dionex). The following gradient was used: eluent A at 100% (0 min), 100% (5 min), 92% (50 min), 0% (55-58 min), (100% (60 min), 100% (75 min). Eluent A was sodium hydroxide (0.1 M) and eluent B was NaAc (0.6 M) in sodium hydroxide (0.1 M). Detection was performed with an ED40 Electrochemical detector (Dionex) with an Au working electrode and an Ag/AgCl reference electrode with a sensitivity of 300 nC. The pulse program used was: +1.0 Volt (0-0.40s); +0.7 Volt (0.41-0.60 s); -0.1 Volt (0.61-1.00 s). Data were integrated using a Turbochrom (Applied Biosystems) data integration system.

Oligosaccharide analysis

(i) synthesis. Acceptor reactions with maltose and isomaltose as acceptor were investigated using 100 mM sucrose and 100 mM acceptor. Products were analyzed with Anion-Exchange chromatography (Dionex) as described above.

(ii) purification. To determine the degree of polymerization (DP) and to purify the oligosaccharides, a BC-200 Ca^{2+} column (at 85 °C; 300 by 7.8 mm; Benson Polymeric) eluted with water (0.2 ml min^{-1}) was used (linear maltoligosaccharides were used to calibrate the system). Detection was done by using a model 830-RI refractive index detector at 40 °C (Jasco).

(iii) characterization. The separate purified oligosaccharides were subjected to enzyme degradation using dextranase from *Penicillium* sp (EC 3.2.1.11, Sigma), which hydrolyzes only α-(1→6) glucosidic bonds (Bourne *et al.*, 1963, Taylor *et al.*, 1995), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, Sigma), which was shown to hydrolyze α-(1→4), α-(1→3) and α-(1→6) linkages at decreasing rates, respectively, to produce glucose from the non-reducing end of linear oligosaccharides (Pazur & Ando, 1960, Pazur & Klepppe, 1962), and α-glucosidase from *Bacillus stearothermophilus* (EC 3.1.2.20 Megazyme, Ireland), which hydrolyzes terminal, α-(1→4) linkages from the non-reducing end of oligosaccharides to produce glucose (Mala *et al.*, 1999). Oligosaccharides (1 $g.l^{-1}$) were incubated with 0.1 U ml^{-1} amyloglucosidase, 66 U ml^{-1} dextranase and 66 U ml^{-1} α-glucosidase. After 30 min, 2 h and 18 h of incubation, samples were withdrawn and products formed (in time) were analyzed by Anion-Exchange chromatography (see above). One endodextranase unit is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmole isomaltose from dextran min^{-1} at 37 °C and pH 6.0. One amyloglucosidase unit is defined as the amount enzyme that hydrolyzes 1 mg of maltose per 3 min at 55 °C and pH 4.5. One α-glucosidase unit is defined as the amount of enzyme that hydrolyzes 1 μmole ρ-nitrophenol-α-glucoside min^{-1} at 40 °C and pH 6.5.

Characterization of the glucans produced

(i) Polymer production. Purified enzyme preparations were incubated with 146 mM sucrose, using the conditions described above under enzyme assays. Glucans produced by *Lb. reuteri* strain BioGaia, and glucans produced with purified recombinant GTFBIO enzyme, were isolated by precipitation with ethanol (van Geel-Schutten *et al.*, 1999).

(ii) Methylation analysis. Polysaccharides were permethylated using methyl iodide and dimsyl sodium ($CH_3SOCH_2^-$-Na^+) in DMSO at room temperature (Hakomori, 1964).

After hydrolysis with 2 M trifluoroacetic acid (1 h, 125 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ (Harris *et al.*, 1984). Mixtures of partially methylated alditol acetates obtained were analyzed by GLC on a CP Sil 5 CB column (25 m × 0.53 mm; Chrompack) and by GLC-mass spectrometry (MS) on a RTX 5 Sil MS (30 m × 0.25 mm; Restek) column (Chaplin, 1982, Jansson *et al.*, 1976).

(iii) NMR spectroscopy. Prior to NMR spectroscopy, samples were dissolved in 99.96 atom % D_2O (Isotec). One-dimensional ^1H NMR spectra were recorded on a 600 MHz Bruker AVANCE NMR spectrometer at a probe temperature of 353K. The HOD signal was suppressed by applying a pressat sequence. Chemical shifts are expressed in ppm by reference to external acetone (δ=2.225). Proton spectra were recorded in 64K data sets, with a spectral width of 8,000 Hz. Resolution enhancement of the spectra was performed with a Lorentzian-to-Gaussian transformation; when necessary, a fifth-order polynomial baseline correction was performed.

(iv) Molecular weights of the glucans. Molecular weight analysis was performed as described previously, using high performance size exclusion chromatography (HPSEC) coupled on-line with a multi angle laser light scattering (MALLS) and differential refractive index detection (Kralj *et al.*, 2002).

RESULTS AND DISCUSSION

Isolation and nucleotide sequence analysis of the putative *Lb. reuteri* strain BioGaia glucosyltransferase gene

Based on sequence homology between conserved regions located in the catalytic core of different *gtf* genes of Gram-positive bacteria, degenerate primers were designed and used for PCR with chromosomal DNA of *Lb. reuteri* strain BioGaia as template (Kralj *et al.*, 2003). The amplified product with the expected size of about 660 bp was ligated into pCR-XL-TOPO (Invitrogen) and transformed to *E. coli* TOP 10. Restriction analysis of ten random clones followed by nucleotide sequence analysis of five clones allowed identification of only one *gtf* gene in LB BIO (*gtfBio*).

In subsequent steps a total of 9419 bp was obtained and sequenced (Fig. 1). Two complete ORFs and two partial ORFs were located on this compiled sequence: ORF1 (5343 bp, Fig. 1), encoding a putative glucosyltransferase (GTFBIO), ORF2 (1269 bp) upstream of ORF1, and ORF3 (243 bp), upstream of ORF2, and ORF4 downstream of *gtfBio* (693 bp). The deduced amino acid sequence of ORF2 showed highest similarity with a reverse transcriptase/maturase family protein from *Clostridium acetobutylicum* (CAC3514; 49 % identity and 71 % similarity within 412 amino acids). The deduced

amino acid sequence of ORF 3 showed highest similarity with a site-specific recombinase from *Streptococcus agalactiae* 2603V/R (SAG1247; 44% identity and 64% similarity within 56 amino acids). The deduced amino acid sequence of ORF4 showed highest similarity with a transposase from *Lb. reuteri* (AF449484; 83% similarity and 91% identity within 231 amino acids). The *gtfBio* gene encodes a putative protein of 1781 amino acids, with a deduced molecular mass of 197,170 Da and a pI of 5.49. The *gtfBio* gene was preceded by a putative ribosomal binding site (GAGGAGGA), localized 6 bp upstream from the TTG-start codon (encoding a formyl methione). According to the consensus promoter sequences described previously for lactobacilli (Pouwels & Leer, 1993), a potential promoter sequence in the upstream sequence of *gtfBio* (40 bp from the start codon) could be identified with a -35 sequence (TTGATA) separated by 22 bp from a -10 sequence (TATTAT).

Two inverted repeats were located 44 and 126 bp downstream from the *gtfBio* termination codon. The first repeat could form a stem (14 bp) loop (8 nucleotides) secondary structure with a ΔG value of –18.4 kcal mol^{-1}. The second repeat could form a stem (18 bp) loop (8 nucleotides) secondary structure with a ΔG value of –28.0 kcal mol^{-1}.

Amino acid sequence alignments of *Lb. reuteri* GTFBIO with other glucosyltransferases

Alignment of the amino acid sequence of GTFBIO with other glucosyltransferases using Blast (Altschul *et al.*, 1990), revealed clear similarities. Highest similarity (68% identity and 80% similarity within 1781 amino acids) at the amino acid level was found with reuteransucrase of *Lb. reuteri* 121 (GTFA), which synthesizes a reuteran with α-(1→4) and α-(1→6) linkages (Kralj *et al.*, 2002). GTFBIO showed also high similarity with GTF180 of *Lb. reuteri* 180 (59% identity and 74 % similarity within 1781 amino acids) and GTFML1 of *Lb. reuteri* ML1 (57% identity and 72% similarity within 1781 amino acids), synthesizing a dextran [mainly α-(1→6) linkages] and mutan [mainly α-(1→3) linkages], respectively (Kralj *et al.*, 2004b). The putative protein structure of GTFBIO was very similar to that of GTFA, GTF180 and GTFML1 containing (i) an N-terminal signal sequence of 38 amino acids, (ii) a relatively large variable N-terminal domain of 705 amino acids (iii) a catalytic domain of 771 amino acids, and a C-terminal domain of 267 amino acids (Fig. 2) (Kralj *et al.*, 2004b).

The deduced N-terminal amino acid sequence of GTFBIO contained a putative secretion peptide with a predicted signal peptidase cleavage site (SPase) between amino acid 38 and 39 (http://www.cbs.dtu.dk/services/SignalP/). Within the deduced N-terminal variable region of GTFBIO, a series of five RDV repeats, R(P/N)DV-x_{12}-SGF-x_{19-22}-R(Y/F)S (x, non-conserved amino acid residue) were found, as previously observed in GTFA, GTF180 and GTFML1 (Kralj *et al.*, 2002, Kralj *et al.*, 2004b).

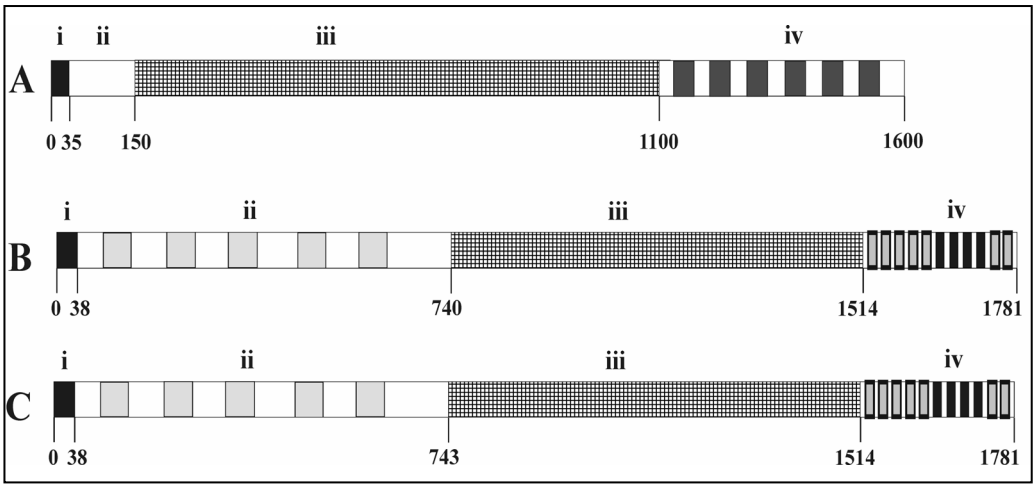

Figure 2. Schematic representation of **A**) glucansucrases in general, **B**) GTFA of *Lb. reuteri* strain 121, and **C**) GTFBIO of *Lb. reuteri* strain BioGaia, showing the four different domains present: i) N-terminal signal sequence; ii) variable region with 5 RDV repeats (light grey boxes); iii) catalytic domain; iv) C-terminal glucan binding domain with four YG-repeating units (dark grey boxes) according to the definition of (Giffard & Jacques, 1994) and seven less conserved YG-repeating units (light grey boxes). Numbers indicate amino acid residue positions.

The relatively short C-terminal domain (glucan binding domain) of GTFBIO contains four YG-repeating units, NDGYYFxxxGxxHºx(G/N)HºHºHº (x, non-conserved amino acid residue; Hº, hydrophobic amino acid residue) according to the definition of (Giffard & Jacques, 1994) and seven YG-repeating units which are less conserved (Fig. 2).

In the deduced amino acid sequence of the catalytic domain of GTFBIO three (putative) catalytic residues could be identified: Asp^{1024}, Glu^{1061} and, Asp^{1133} (Fig. 3). In the region downstream of the (putative) catalytic nucleophile Asp^{1024}, ^{1024}DAPDNI, GTFBIO differs in 2 out of 5 amino acids conserved in virtually all studied GTF enzymes (Fig. 3). In GTFBIO, Pro^{1026} is found in a position where a conserved Val is present in all other glucansucrases (except GTFA of *Lb. reuteri* 121, which also possesses a Pro residue at this position) (Fig. 3). Another conserved amino acid substitution in this region of GTFBIO, Ile^{1029} (instead of Val), was also found in amylosucrase, a glucosyltransferase from *Neisseria polysaccharea* synthesizing an α-(1→4) glucan (De Montalk *et al.*, 1999), CD2 of DSRE of *Ln. mesenteroides* NRRL-B1299 responsible for the synthesis of α-(1→2) linkages (Bozonnet *et al.*, 2002) and GTFA of *Lb. reuteri* 121 (Kralj *et al.*, 2002). This region in GTFBIO is identical to that in GTFA of *Lb. reuteri* 121, which also synthesizes a reuteran, and thus may be responsible for the α-(1→4) bond specificity in both enzymes (Fig. 3).

	Main α-linkages in glucan polymer		**A**		**B**		**C**
			∀ □□ ‡		⇓□□□		♦ ‡
GTFBIO	1→4	1016	ANFDSVRVDAPDNIDADLMNI	1056	HINILEDWNSSDPEY	1126	YSFIRAHDNNSQDQI
GTFA	1→4 / 1→6	1016	ANFDSVRVDAPDNIDADLMNI	1056	HINILEDWNHADPEY	1126	YSFVRAHDNNSQDQI
GTF180	1→6	1017	ANFDGIRVDAVDNVDVDLLSI	1058	HINILEDWGWDDPAY	1129	YNFVRAHDSNAQDQI
GTFML1	1→3	1017	ANFDSIRVDAVDNVDADLLDI	1058	HINILEDWGGQDPYY	1125	YSFIRAHDNGSQDDI
GTFB	1→3	443	ANFDSIRVDAVDNV**D**ADLLQI	484	HLSILEA**W**SDNDTPY	555	YSFIRAHDSEVQ**D**LI
GTFD	1→6	457	ANFDGVRVDAVDNV**N**ADLLQI	498	HLSILEAWSDNDPQY	577	YIFIRAHDSEVQ**T**VI
GTFI	1→3	445	ANFDSIRVDAVDNVDADLLQI	486	HVSIVEAWSDNDTPY	557	YSFARAHDSEVQ**D**LI
GTFS	1→6	388	ANFDGVRVDAVDNVNADLLQI	429	HLSILEAWSGNDNDY	470	YVFIRAHDSEVQTRI
GTFJ	1→3	463	ANFDGIRVDAVDNVDADMLQL	504	HISVLEAWSLNDNHY	605	YVFIRAHDNNVQDII
GTFK	1→6	453	AHFDGIRVDAVDNVSVDMLQL	494	NISILEAWSHNDPYY	575	YLFVRAHDSEVQTVI
DSRB	1→6	525	ANFDGIRVDAVDNVDADLLQI	566	HLSILEDWSHNDPEY	637	YSFVRAHDSEVQTVI
DSRS	1→6	543	ANFDGIRVDAVDNVDADLLQI	584	HLSILEDWSHNDPLY	655	YSFVRAHDSEVQ**T**VI
ASR	1→6 / 1→3	626	ANFDGIRVDAVDNVDADLLKI	667	HLSILEDWNGKDPQY	759	YSFVRAHDYDAQDPI
DSRE CD1	1→6	519	ANFDGYRVDAVDNVDADLLQI	560	HISILEDWDNNDSAY	631	YAFIRAHDSEVQTVI
DSRE CD2	1→2	2202	ANFDSIRIDAVDFIHNDTIQR	2243	HISLVEAGLDAGTST	2315	YSIIHAHDKGVQEKV
			*:**. *:** * : * :.		::.::* .		* : :*** * :

Figure 3. Amino acid sequence alignment of highly conserved stretches (A, B, C) in catalytic domains of dextran-, mutan-, alternan- and reuteransucrases of lactic acid bacteria (also see (Monchois *et al.*, 1999d)). GTFBIO, *Lb. reuteri* BioGaia (this study); GTFA, *Lb. reuteri* 121 (Kralj *et al.*, 2002); GTF180 of *Lb. reuteri* 180 (Kralj *et al.*, 2004b); GTFML1 of *Lb. reuteri* ML1 (Kralj *et al.*, 2004b); GTFB, *S. mutans* GS5 (Shiroza *et al.*, 1987); GTFD, *S. mutans* GS5 (Honda *et al.*, 1990); GTFI, *S. downei* MFe28 (Ferretti *et al.*, 1987); GTFS, *S. downei* MFe28 (Gilmore *et al.*, 1990); GTFJ, *Streptococcus salivarius* ATCC 25975 (Giffard *et al.*, 1991); GTFK, *S. salivarius* ATCC 25975 (Giffard *et al.*, 1993); DSRB, *Ln. mesenteroides* NRRL B-1299 (Monchois *et al.*, 1998a); DSRS, *Ln. mesenteroides* NRRL B-512F (Monchois *et al.*, 1997); ASR, *Ln. mesenteroides* NRRL B-1355 (Arguello-Morales *et al.*, 2000); DSRE CD1 and CD2, *Ln. mesenteroides* NRRL-B1299(Bozonnet *et al.*, 2002);***** , identical residue; **:** , highly conserved residue; **.** , conserved residue; ∀, putative nucleophile (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); ⇓, putative acid/base catalyst (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); ♦, putative residue stabilizing the transition state (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); □, putative acceptor binding / glycosyl transfer sites (MacGregor *et al.*, 1996); ‡, residues involved in glucan solubility and structure determination as shown for three different glucansucrases, mutated amino acids shown in bold face (Shimamura *et al.*, 1994, Remaud-Simeon *et al.*, 2000, Monchois *et al.*, 2000a); Tryptophan residue important for activity, shown in bold face (Tsumori *et al.*, 1997).

The region following the putative acid base catalyst E1061 (putative acceptor region) is not highly conserved among glucansucrases (Monchois *et al.*, 1999d). Mutations in the region following the catalytic glutamate in the CGTase enzymes of *B. circulans* 251 and *Thermoanaerobacterium thermosulfurigenes* strain EM1, resulted in an increase in hydrolytic activity and decrease of cyclization activity (van der Veen *et al.*, 2001, Leemhuis *et al.*, 2002). Immediately following the motif ^{1061}E(A/D)W(S/N), two serine residues are found in GTFBIO (Fig. 3), whereas GTFA of *Lb. reuteri* 121 possesses a histidine and an alanine at the corresponding positions, respectively. We speculate that differences in this (putative) acceptor site region between GTFBIO and GTFA may at

least partly explain either the larger amount of α-(1→4) bonds synthesized by GTFBIO in its products (see below) and/or its higher hydrolytic activity (see below).

The region following D1133 (transition state stabilizer) in GTFBIO differs from the sequence ^{1133}DSEVQTVI, conserved in many glucansucrases from *Streptococcus*, *Leuconostoc* and *Lactobacillus* (Fig. 3) (Monchois *et al.*, 1999d, Kralj *et al.*, 2003). In GTFBIO as well as in GTFA, an original tripeptide NNS is found immediately downstream of this catalytic Asp. Also GTF180 and GTFML1 contain both an original tripeptide at this position, SNA and NGS, respectively. Finally, also alternansucrase and CD2 of DSRE of *Ln. mesenteroides* NRRL-B1299 contain both an original tripeptide at this position, YDA and KGV, respectively (Fig. 3) (Bozonnet *et al.*, 2002). Mutational evidence is available that the region following this catalytic Asp^{1133}, is important in glucan structure determination in GTF enzymes. Mutations in an amino acid residue located five amino acids C-terminal of the catalytic Asp^{1133}, T589D in GTFD of *S. mutans* GS5, D567T in GTFB of *S. mutans* GS5 (Shimamura *et al.*, 1994), T667R in DSRS of *Ln. mesenteroides* (Remaud-Simeon *et al.*, 2000), and D569X in GTFI of *S. downei* (Monchois *et al.*, 2000a), showed its involvment in glucan solubility and structure determination (Fig. 3). This residue itself is not completely conserved in glucansucrases but usually an Asp or Thr is found at this position (Fig. 3). The presence of an original tripeptide NNS in GTFA as well as GTFBIO, may partly explain the unique structure of the glucans synthesized by both enzymes containing high amounts of α-(1→4) linkages.

Purification, pH and temperature optima

Lb. reuteri BioGaia GTFBIO(-ΔN) enzymes expressed in *E. coli* were purified to homogeneity. The predicted M_r's of the N-terminal deletion mutant (117 KDa, without signal sequence and N-terminal variable region) and full-length protein (194 KDa, without signal sequence) was in agreement with the results obtained by SDS-PAGE analysis (data not shown). In order to define the best conditions for subsequent kinetic studies, the pH and temperature optima of GTFBIO were examined. The pH optima for both the hydrolyzing and transferase activities were at pH 5.0 (Data not shown). The temperature optima for both reactions were 35 °C (Data not shown). The N-terminal deletion mutant (GTFBIO-ΔN) showed the same pH and temperature optima (Data not shown). The pH optimum of GTFBIO(-ΔN) was similar to that of GTFA of *Lb. reuteri* 121 (pH 4.7). However, the temperature optimum of GTFBIO was 15 °C lower than that of GTFA (50 °C) (Kralj *et al.*, 2004d).

Kinetic studies with GTFBIO(-ΔN)

(i) Kinetic parameters. In the presence of sucrose, GTFBIO displayed Michaelis-Menten type of kinetics for hydrolysis (V_G) and for total enzyme activity (V_F; Table 1A). Transferase activity (initial rates; V_F-V_G) was observed only at sucrose concentrations

above 10 mM. GTFBIO thus favours hydrolysis at low sucrose concentrations and polymerization at higher sucrose concentrations. GTFA clearly displayed transferase activity (initial rates) at sucrose concentrations below 10 mM (Kralj *et al.*, 2004d). GTFBIO-ΔN showed similar kinetics as full-length GTFBIO (data not shown). Affinity for the substrate sucrose in the hydrolysis reaction was similar for both enzymes. Deletion of the N-terminal variable domain in GTFA resulted in more drastic changes in enzyme activity, especially resulting in a strongly increased transferase activity and an approximately 2 times lower turnover rate for the hydrolysis reaction (Kralj *et al.*, 2004d). Deletion of the N-terminal variable domain in GTFBIO did not result in an increase in its transferase activity. However, the hydrolysis reaction turnover rates (*k*cat s^{-1}) for GTFBIO-ΔN for were about 1.5 times higher than for full-length GTFBIO (data not shown).

Table 1. Comparison of the kinetic properties and product spectra of two reuteransucrase enzymes, GTFBIO of *Lb. reuteri* strain BioGaia and GTFA of *Lb. reuteri* 121. GTFBIO-ΔN and full length GTFBIO displayed similar kinetics properties and product spectra (data not shown). **A**) Kinetic parameters were determined using 15 different concentrations of sucrose (0.25-100 mM). **B**) Product spectra obtained with sucrose (100 mM), or sucrose and maltose (100 mM each), or sucrose and isomaltose (100 mM each). † Oligosaccharide yield is expressed as a percentage of the total amount of acceptor used in the incubation. * No transferase activity could be determined for GTFBIO: only hydrolysis occurred at sucrose concentrations below 10 mM. # Hydrolysis reaction of GTFA was fitted with the Michaelis-Menten equation with substrate inhibition. Ki^G = 111.0± 12.0 mM). ‡ Data from (Kralj *et al.*, 2004d).

A

Enzyme	Total Activity		Transferase activity		Hydrolysis activity	
	$V\text{max}^F$ (U.mg^{-1})	$K\text{m}^F$	$V\text{max}^{F\text{-}G}$ (U.mg^{-1})	$K\text{m}^{F\text{-}G}$	$V\text{max}^G$ (U.mg^{-1})	$K\text{m}^G$
GTFA	25.5 ± 0.4	0.9 ± 0.1	11.3 ± 1.2	4.6 ± 0.8	20.3 ± 0.5#	1.1 ± 0.1
GTFBIO	13.5 ± 0.4	1.2 ± 0.1	-*	-*	13.9 ± 0.3	1.5 ± 0.1

B

	Reuteran synthesis (%)	Isomaltose synthesis (%)	Leucrose synthesis (%)	Hydrolysis (%)	Acceptor reaction oligosaccharide yield (%)† in the presence of:	
					Maltose	Isomaltose
GTFA‡	73.7 ± 3.6	1.6 ± 0.2	2.1 ± 0.1	22.7 ± 3.2	59.6 ± 3.9	28.3 ± 2.1
GTFBIO	35.9 ± 4.3	4.0 ± 0.2	5.7 ± 0.2	54.5 ± 3.9	63.4 ± 0.8	50.8 ± 0.9

(ii) Effect of maltose on initial GTFBIO activity. The stimulating effect of maltose on initial GTFBIO total activity (seven fold; $V_{F,}$ 95.4 ± 2.2) was also observed for GTFA (3.5 fold; $V_{F,}$ 97.7 ± 0.9) (Kralj *et al.*, 2004d).

Product analysis

(i) Product spectrum from sucrose. Compared to GTFA, GTFBIO converted less sucrose into glucan but produced slightly higher amounts of isomaltose and leucrose. GTFBIO action on sucrose resulted in considerably more glucose release of sucrose (>54 % of sucrose; Table 1B) compared to GTFA (22.7 % of sucrose; Table 1B). Also two unknown products eluted after 36 and 54 min, respectively. GTFBIO-ΔN showed a similar product spectrum as GTFBIO (data not shown).

(ii) Enzymatic analysis of oligosaccharides structure synthesized with maltose and isomaltose as acceptor substrates. The major oligosaccharide product synthesized by both GTFA and GTFBIO with sucrose and maltose as acceptor reaction substrate eluted at exactly the same position (43.5 min; Dionex analysis; Fig. 4A, B) as panose (α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose) (Kralj *et al.*, 2004d). This indicated that an α-(1→6) linkage was formed at the non-reducing end of maltose resulting in synthesis of an oligosaccharide with a degree of polymerization (DP) of 3. This oligosaccharide was purified on basis of its size using HPLC (see methods) (Kralj *et al.*, 2004d). Dionex analysis of products formed upon its enzymatic degradation in time, to glucose and (maltose) by amyloglucosidase and alpha glucosidase, confirmed its identity as panose (Table 2).

Table 2. Enzymatic degradation followed by Dionex analysis of products formed by the reuteransucrase enzymes GTFBIO and GTFA, from sucrose and different acceptor substrates. (G = glucose, G2 = maltose, IG2 = isomaltose, PAN = panose, IPAN = isopanose, 1,6-IPAN = α-D-glucopyranosyl-α-(1→6)-α-D-glucopyranosyl-α-[(1→4)-α-D-glucopyranosyl-(α-(1→6)]-D-glucose. * fast degradation in time, † slow degradation in time, ‡ no degradation, # the DP4 product formed by GTFA was analyzed, because it was synthesized only in relatively small amounts by GTFBIO.

DP oligosaccharide (acceptor used)	Dextranase	Amyloglucosidase	Alpha glucosidase	ID, oligo structure
3 (maltose)	-‡	G + G2†	G†	PAN
3 (isomaltose)	-‡	G + IG2*	G + IG2*	IPAN
4 (isomaltose)#	-‡	G + IG2 + IPAN†	G†	1,6-IPAN

The major oligosaccharide synthesized by both GTFA and GTFBIO with sucrose and isomaltose as acceptor reaction substrate eluted after 34 min. Its degree of polymerization was 3, as determined by HPLC analysis (Kralj *et al.*, 2004d). Its identity (α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-α-glucose; isopanose) was further deduced from the products formed upon its degradation to isomaltose and glucose by amyloglucosidase and alpha glucosidase (Table 2). Degradation of isopanose by

amyloglucosidase and alpha glucosidase was much faster than degradation of panose. This confirms that an α-(1→4) linkage (cleavage of α-1→4 linkages from the non-reducing end is preferred by both enzymes; see methods) had been formed at the non-reducing end of isomaltose, resulting in synthesis of isopanose (Table 2).

Besides the DP 3 oligosaccharide, small amounts of a DP 4 oligosaccharide, eluting after 46 min (as identified by HPLC analysis), was synthesized by GTFBIO. GTFA synthesized larger amounts of this DP 4 oligosaccharide (Fig 4C, D) (Kralj et al., 2004d). Therefore, the DP 4 oligosaccharide synthesized by GTFA was used for HPLC purification and subsequent enzymatic analysis. This oligosaccharide was degraded (slowly) by amyloglucosidase to glucose, isomaltose and isopanose. Degradation of this oligosaccharide by alpha glucosidase was poor. This suggest that an α-(1→6) linkage is present at its non-reducing end (see above). Degradation by dextranase was not possible indicating that α-(1→4) linkage is present in the middle of this oligosaccharide (another DP4 oligosaccharide with an α-(1→6) linkage in the middle was cleaved by dextranase; data not shown). Isopanose is one of the degradation products this suggest that the isopanose formed by GTFA is used as acceptor reaction substrate to form α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose; α-(1→6)-isopanose.

(iii) Comparison of the (amounts of) oligosaccharides synthesized by GTFA and GTFBIO. Analysis showed that (see above), in the presence of maltose, GTFA and GTFBIO formed panose (α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose) as most abundant acceptor reaction product (from 100 mM sucrose and 100 mM maltose, GTFA and GTFBIO synthesized approximately 44 mM and 33 mM panose, respectively; data not shown; Fig. 4A, B). Also relatively large amounts of maltotriose (17 mM; data not shown; Fig. 4B) were synthesized by GTFBIO, indicating that an α-(1→4) linkage was formed (most probably) at the non-reducing end of maltose. GTFA of *Lb. reuteri* 121 synthesized higher amounts of panose (Kralj et al., 2004d) and lower amounts of maltotriose (5 mM, Fig. 4A). Oligosaccharide yields for GTFBIO and GTFA with maltose as acceptor substrate were rather similar (~60 %). Maltose thus appears to be an equally good acceptor reaction substrate for oligosaccharide synthesis by both GTFBIO and GTFA (Table 1B).

With isomaltose as acceptor, GTFBIO formed low amounts of isomaltotriose and isomaltotetraose, plus an abundant oligosaccharide product (Fig. 4D) with a DP of 3 (identified by enzymatic degradation as isopanose, see above). GTFBIO synthesized approximately 2.5 times more isopanose than GTFA (Fig. 4C, D). Besides isopanose, GTFBIO synthesized also minor amounts of a DP 4 oligosaccharide from sucrose and isomaltose, identified by enzymatic degradation as α-(1→6)-isopanose (Table 2). GTFA

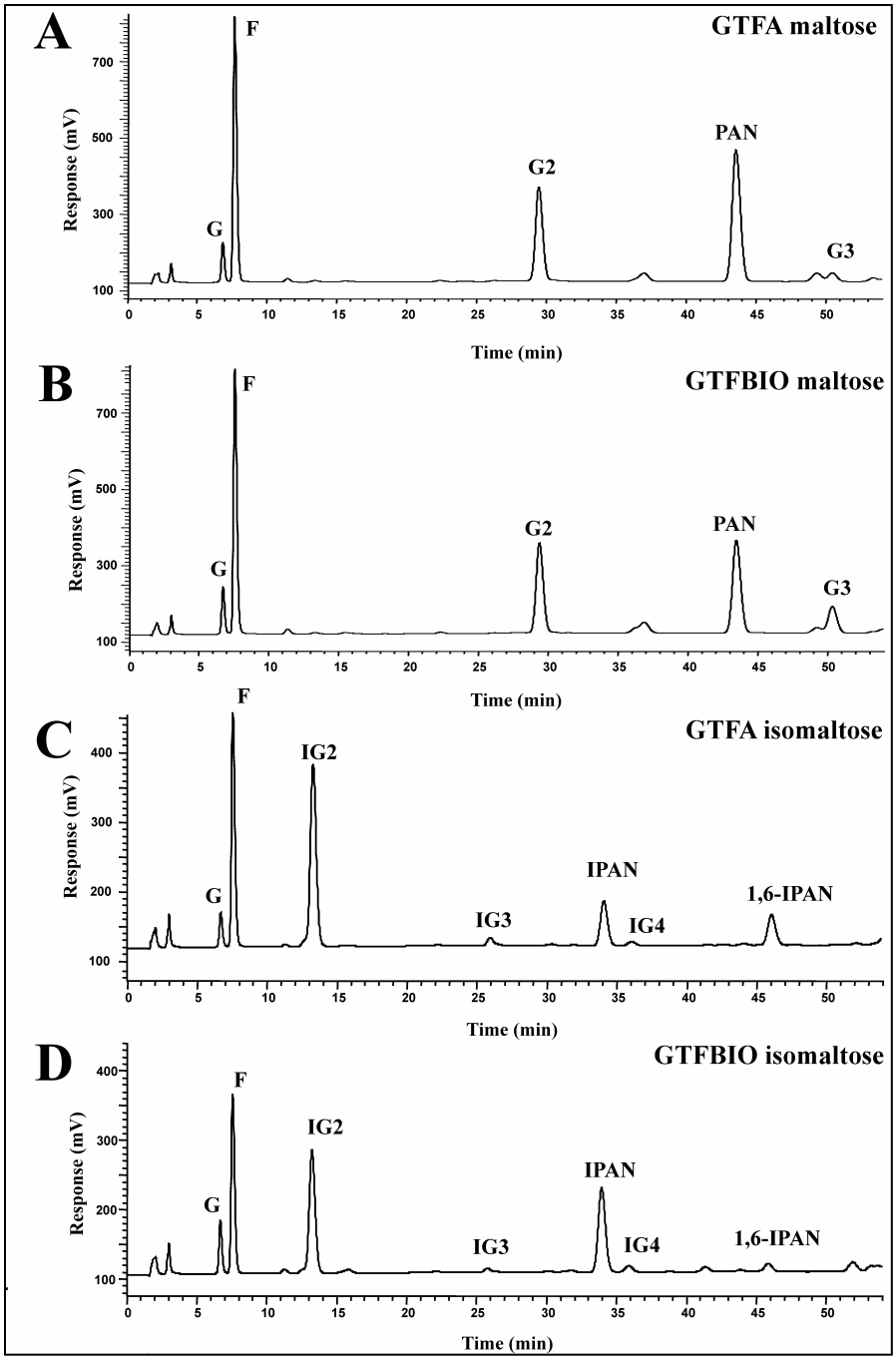

Figure 4. Dionex analysis (as described in materials and methods) of GTFBIO and GTFA (data from (Kralj *et al.*, 2004d)) acceptor reaction products. **A**) Products formed upon incubation of 30 nM GTFA enzyme with 100 mM sucrose and 100 mM maltose for 60 h; **B**) Products formed upon incubation of 30 nM GTFBIO enzyme with 100 mM sucrose and 100 mM maltose for 60 h; **C**) Products formed upon incubation of 30 nM GTFA enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h; **D**) Products formed upon incubation of 30 nM GTFBIO enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h. (F = fructose, G = glucose, G2 = maltose, G3 = maltotriose, IG2 = isomaltose, IG3 = isomaltotriose, IG4 = isomaltotetraose, PAN = panose, IPAN = isopanose, 1,6-IPAN = α-D-glucopyranosyl-α-(1→6)-α-D-glucopyranosyl-α-[(1→4)-α-D-glucopyranosyl-(α-(1→6)]-D-glucose.

synthesized significantly larger amounts of α-(1→6)-isopanose (Fig. 4C,D) (Kralj *et al.*, 2004d). The GTFBIO yield of oligosaccharides with isomaltose as acceptor substrate (50.8 %) is double the GTFA yield (28.3 %) (Table 1B). Isomaltose thus is a two times better acceptor reaction substrate for oligosaccharide synthesis by GTFBIO than for GTFA. GTFBIO-ΔN displayed a similar product distribution with maltose and isomaltose as acceptor reaction substrates (data not shown).

Compared to GTFA, GTFBIO thus introduced more α-(1→4) linkages in its acceptor substrates, yielding larger amounts of maltotriose from maltose and larger amounts of isopanose from isomaltose, and only minor amounts of the DP 4 oligosaccharide α-(1→6)-isopanose (Fig. 4). The ability of GTFBIO to synthesize an α-glucan with large amounts of α-(1→4) linkages (see below) thus also is reflected in its oligosaccharide product spectrum.

Analysis of the glucans produced by *Lb. reuteri* BioGaia culture supernatants and purified recombinant GTFBIO(-ΔN) enzymes

Purified recombinant GTFBIO and GTFBIO-ΔN enzymes and supernatants of sucrose grown cultures of *Lb. reuteri* BioGaia, incubated with sucrose produced high molecular weight glucans.

Table 3. Methylation analysis, NMR (see also fig. 5) and molecular masses of the glucans produced from sucrose by *Lb. reuteri* strain BioGaia culture supernatants and by the purified recombinant GTFBIO, GTFBIO-ΔN and GTFA enzymes. ND = not determined.

Type of glucosyl unit	**Methylation (%)**			
	***Lb. reuteri* strain BioGaia**	**GTFBIO**	**GTFBIO-ΔN**	**GTFA**
Terminal	9	5	5	7
α-(1→4)	69	67	69	47
α-(1→6)	11	13	14	35
α-(1→4,6)	13	15	13	11
		NMR (%)		
α-(1→6) [~5.0 ppm]	19	ND	21	43
α-(1→4) [~5.3 ppm]	81		79	57
		Molecular mass (1×10^6 Da)		
	28	45	42	45

Using HPSEC-MALLS, the average molecular mass of the glucan produced by LB BIO was determined as $28\cdot10^6$ Da. The molecular masses of the glucans produced by the

purified GTFBIO and GTFBIO-ΔN were $45 \cdot 10^6$ Da and $42 \cdot 10^6$ Da, respectively (Table 3). The identical nature of the glucans was confirmed by methylation analysis (Table 3). The ^{1}H-NMR spectra of the glucans produced by LB BIO culture supernatants and by the purified recombinant GTFBIO-ΔN enzyme, were virtually identical (Table 3, Fig. 5A,B). Comparison of both ^{1}H-NMR spectra with that of the reuteran produced by the *Lb. reuteri* 121 GTFA enzyme (Table 3, Fig. 5C) (van Geel-Schutten *et al.*, 1999, Kralj *et al.*, 2002) showed that both glucans consist of α-(1→4) [~5.3 ppm] and α-(1→6) [~5.0 ppm] linked glucopyranosyl units. Due to poor resolution of the spectra it was not possible to trace the terminal and α-(1→4,6) linked residues present as indicated by the methylation analysis (Table 3).

NMR (Table 3, Fig. 5) and methylation analysis (Table 3) clearly show that GTFBIO and GTFA synthesize different reuteran products. GTFBIO reuteran contains approximately 20% more α-(1→4) glucosidic linkages and 20% less α-(1→6) glucosidic linkages. The degree of branching of the GTFBIO and GTFA synthesized reuterans (amount of both terminal and α-(1→4,6) glucosyl units) is rather similar as seen from the methylation analysis (Table 3). The precise structures of both reuterans remain to be elucidated.

Conclusion

This paper reports the molecular and biochemical characterization of a novel *Lb. reuteri* BioGaia reuteransucrase gene (*gtfBio*), and the novel reuteransucrase enzyme (GTFBIO) encoded. A detailed analysis showed that GTFBIO produces a unique, soluble reuteran, a glucan containing the largest amount of α-(1→4) glucosidic linkages (~ 70%) reported to date. This glucan also contains α-(1→6) linked glucosyl units and 4,6-disubstituted α-glucosyl units at the branching points. Expression of *gtfBio* in *E. coli* yielded an active glucosyltransferase enzyme that was purified and shown to synthesize virtually the same soluble glucan with respect to binding type. The sizes of the glucans synthesized by recombinant GTFBIO(-ΔN) were slightly larger than the glucan synthesized by LB BIO. When incubated with 100 mM sucrose the product spectrum of GTFBIO reuteransucrase showed a relatively low glucan yield (< 36% of sucrose consumed), whereas the hydrolysis yield was high (> 54% of sucrose consumed). The GTFBIO, GTFA, GTF180 and GTFML1 structures and amino acid sequences are highly similar (Kralj *et al.*, 2004b) (~70 % identity, > 80 % similarity) but they synthesize glucans with different glucosidic linkages (mainly α-(1→4), α-(1→4)/α-(1→6), mainly α-(1→6) and mainly α-(1→3) linkages, respectively. These enzymes thus are very interesting for structure/function studies aiming to identify amino acid residues responsible for glucosidic bond specificity. Furthermore, GTFA and GTFBIO are

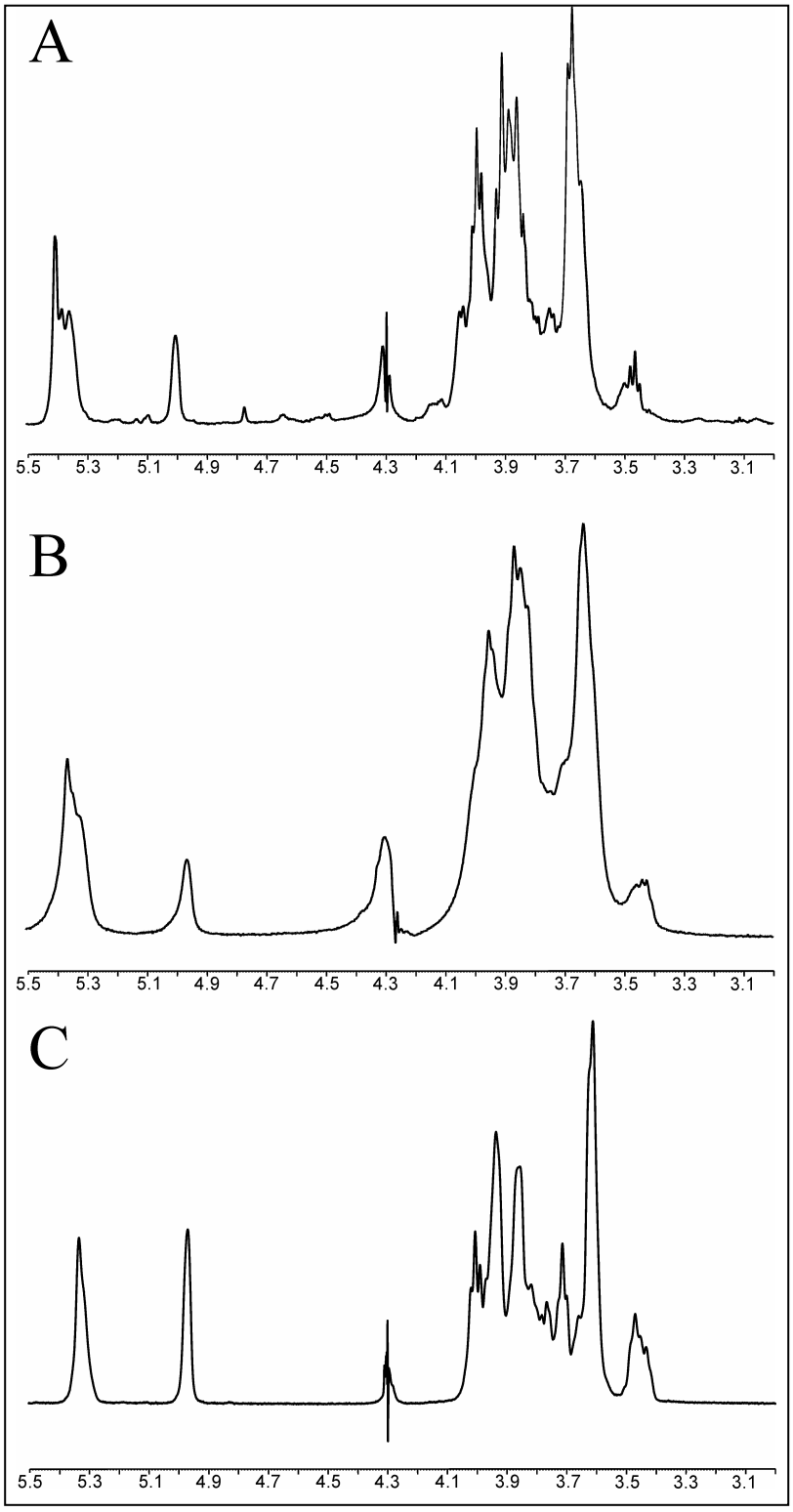

Figure 5. 600-MHz ^{1}H-NMR spectra of the glucans produced by (**A**) *Lb. reuteri* strain BioGaia GTFBIO culture supernatants, by (**B**) purified recombinant GTFBIO-ΔN protein and by (**C**) purified recombinant GTFA protein of *Lb. reuteri* strain 121 (Kralj *et al.*, 2004d) recorded in D_2O at 80°C. Chemical shifts are given in parts per million relative to the signal of internal acetone (δ=2.225).

interesting candidates to examine structural differences responsible for hydrolysis/transferase activity ratios.

ACKNOWLEDGEMENTS

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Chapter 5

Rational transformation of *Lactobacillus reuteri* 121 reuteransucrase into a dextransucrase: analysis of mutant enzymes and their glucan and oligosaccharide products

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SUMMARY

Glucansucrase or glucosyltransferase (GTF) enzymes of lactic acid bacteria display high sequence similarity but catalyse synthesis of different α-glucans (e.g. dextran, mutan, alternan and reuteran) from sucrose. Reuteran has recently been characterized as a novel α-glucan with mainly α-(1→4) glucosidic linkages produced only by reuteransucrase enzymes of *Lactobacillus reuteri* strains.

The variations in glucosidic linkage specificity observed in products of different glucansucrase enzymes appear to be based on relatively small differences in amino acid sequences in their sugar-binding acceptor subsites. This notion was derived from mutagenesis of amino acids of GTFA (reuteransucrase) from *Lb. reuteri* strain 121 putatively involved in acceptor substrate binding. A triple amino acid mutation (N1134S:N1135E:S1136V) in a region immediately next to the catalytic Asp1133 (putative transition state stabilizing residue) converted GTFA from a mainly α-(1→4) (~45%, reuteran) to a mainly α-(1→6) (~80%, dextran) synthesizing enzyme. The subsequent introduction of mutation P1026V:I1029V, involving two residues located in a region next to the catalytic Asp1024 (nucleophile), resulted in the synthesis of an α-glucan containing only a very small percentage of α-(1→4) glucosidic linkages (~5%) and a further increased percentage of α-(1→6) glucosidic linkages (~85%). This changed glucosidic linkage specificity was also observed in the oligosaccharide products synthesized by the different mutant GTFA enzymes from (iso)maltose and sucrose.

Amino acids crucial for glucosidic linkage type specificity of reuteransucrase have been identified in this report. The data show that a combination of mutations in different regions of GTF enzymes influences the nature of both the glucan and oligosaccharide products. The amino acids involved most likely contribute to sugar-binding acceptor subsites in glucansucrase enzymes.

INTRODUCTION

Glucansucrase or glucosyltransferase (GTF) enzymes (EC 2.4.1.5) of lactic acid bacteria (LAB) are able to synthesize a diversity of α-glucans with α-(1→6) (dextran by dextransucrases (DSR), mainly found in *Leuconostoc*), α-(1→3) (mutan by mutansucrase, mainly found in *Streptococcus*), alternating α-(1→3) and α-(1→6) (alternan by alternansucrase (ASR), only reported in *Leuconostoc mesenteroides*), α-(1→2) (only reported in *Ln. mesenteroides*), or α-(1→4) (reuteran by reuteransucrase, only reported in *Lactobacillus reuteri*) glucosidic bonds (Monchois *et al.*, 1999d, Kralj *et al.*, 2002, Bozonnet *et al.*, 2002, Kralj *et al.*, 2004b) Two novel types of α-glucans, and the

glucansucrase enzymes involved, have been characterized only recently. DSRE of *Ln. mesenteroides* NRRL B-1299 synthesizes a unique glucan with α-(1→2) glucosidic branch linkages (Bozonnet *et al.*, 2002). Reuteransucrase (GTFA) from *Lactobacillus reuteri* 121 is an 1,781 amino acid enzyme that synthesizes a unique soluble glucan polymer (reuteran) with mainly α-(1→4) glucosidic linkages and significant amounts of α-(1→6) and α-(1→4,6) glucosidic linkages (van Geel-Schutten *et al.*, 1999, Kralj *et al.*, 2002).

Two different reactions are catalyzed by glucansucrase enzymes, depending on the nature of the acceptor substrate: i) hydrolysis, when water is used as acceptor; ii) glucosyl transfer (transferase), which can be divided in: a) polymerization, when the growing glucan chain is used as acceptor, and b) oligosaccharide synthesis, when alternative acceptors are used (e.g. oligosaccharides such as maltose, isomaltose). Where studied, the linkage specificity of glucansucrases is conserved in oligosaccharide synthesis (Dols *et al.*, 1997, Cote & Robyt, 1982, Robyt & Walseth, 1978, Kralj *et al.*, 2004a), and oligosaccharides are elongated at their non-reducing end (Dols *et al.*, 1997, Arguello Morales *et al.*, 2001, Monchois *et al.*, 2000a, Mukasa *et al.*, 2000).

All glucansucrases possess a common pattern of structural organization: their N-terminal end starts with (i) a signal peptide, followed by (ii) a highly variable stretch, (iii) a highly conserved catalytic or sucrose binding domain, and (iv) a C-terminal domain composed of a series of tandem repeats (Monchois *et al.*, 1999d). More than 30 different glucansucrases have been at (least partly) (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html) characterized (members of glycoside hydrolase family 70; GH70), but no detailed structural information is yet available.

The 3D structures and catalytic mechanism have been elucidated for several enzymes of glycoside hydrolase family 13 (GH13) (McCarter & Withers, 1994, Uitdehaag *et al.*, 1999), including that of amylosucrase, the only enzyme of family GH13 that uses sucrose as substrate to synthesize α-glucan polymers containing α-(1→4) glucosidic linkages (Albenne *et al.*, 2004, Skov *et al.*, 2001). Most members of family GH13 act on starch, e.g. α-amylase and cyclodextrin glycosyltransferase (CGTase) and hydrolyse and/or synthesize α-(1→4) and α-(1→6) glucosidic linkages (van der Veen *et al.*, 2000).

Secondary-structure predictions revealed that the catalytic domains of GTF enzymes possess a $(\beta/\alpha)_8$-barrel structure similar to members of family GH13. The presence of this $(\beta/\alpha)_8$-barrel structure was supported by circular dichroism experiments (Monchois *et al.*, 1999b). The core of the proteins belonging to family GH13 constitutes 8 β-sheets alternated with 8 α-helices. In GTFs, however, this $(\beta/\alpha)_8$ barrel structure is circularly permuted (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997, Devulapalle *et al.*, 1997). Therefore, GTF enzymes are classified in family GH70 (Henrissat & Bairoch, 1996). Also the four conserved regions (I-IV) identified in members of the α-amylase family GH13 (Svensson, 1994) can be found in glucansucrases. However, as a consequence of the

circular permutation, region I occurs C-terminal of region II-IV in glucansucrase enzymes. We have adopted the same numbering for the conserved regions in glucansucrase enzymes (Fig 1). Questions about the exact catalytic mechanism and structure/function relationships of these intriguing enzymes remain to be answered (Monchois *et al.*, 1999d, Albenne *et al.*, 2004). The largely similar structural features of

Enzyme	Main α-linkages in glucan polymer	**Region II**		**Region III**		**Region IV**	
			∀ □□ ‡		⇓□□□		♦ ‡ ‡
GTFA	1→4	1016	ANFDSVRV***D***A**P**DN**I**DADLMNI	1056	HINIL***E***DWN**HA**DPEY	1126	YSFVRAH***D*****NNS**QDQIQNA
GTFBIO	1→4 / 1→6	1016	ANFDSVRVDAPDNIDADLMNI	1056	HINILEDWNSSDPEY	1126	YSFIRAHDNNSQDQIQNA
GTF180	1→6	1017	ANFDGIRVDAVDNVDVDLLSI	1058	HINILEDWGWDDPAY	1129	YNFVRAHDSNAQDQIRQA
GTFML1	1→3	1017	ANFDSIRVDAVDNVDADLLDI	1058	HINILEDWGGQDPYY	1125	YSFIRAHDNGSQDDIKRA
GTFB	1→3	443	ANFDSIRV***D***AVDNV**D**ADLLQI	484	HLSILEA**W**SDNDTPY	555	YSFIRAHDSEVQ**D**LIA**D**I
GTFD	1→6	457	ANFDGVRVDAVDNV**N**ADLLQI	498	HLSILEAWSDNDPQY	577	YIFIRAHDSEVQ**T**VIAKI
GTFI	1→3	445	ANFDSIRV***D***AVDNVDADLLQI	486	HVSIV***E***AWSDNDTPY	557	YSFARAH***D***SEVQ**D**LIRDI
GTFS	1→6	388	ANFDGVRVDAVDNVNADLLQI	429	HLSILEAWSGNDNDY	470	YVFIRAHDSEVQTRIAKI
GTFJ	1→3	463	ANFDGIRVDAVDNVDADMLQL	504	HISVLEAWSLNDNHY	605	YVFIRAHDNNVQDIIAEI
GTFK	1→6	453	AHFDGIRVDAVDNVSVDMLQL	494	NISILEAWSHNDPYY	575	YLFVRAHDSEVQTVIADI
DSRB	1→6	525	ANFDGIRVDAVDNVDADLLQI	566	HLSILEDWSHNDPEY	637	YSFVRAHDSEVQTVIAQI
DSRS	1→6	543	ANFDGIRV***D***AVDNVDADLLQI	584	HLSILEDWSHNDPLY	655	YSFVRAHDSEVQ**T**VIAQI
ASR	1→6 / 1→3	626	ANFDGIRVDAVDNVDADLLKI	667	HLSILEDWNGKDPQY	759	YSFVRAHDYDAQDPIRKA
DSRE CD1	1→6	519	ANFDGYRVDAVDNVDADLLQI	560	HISILEDWDNNDSAY	631	YAFIRAHDSEVQTVIAQI
DSRE CD2	1→2	2202	ANFDSIRIDAVDFIHNDTIQR	2243	HISLVEAGLDAGTST	2315	YSIIHAHDKGVQEKVGAA
			*:**. *:** * : * :.		::.::* .		* : :*** * :

Figure 1. Amino acid sequence alignment of (highly) conserved regions (II, III, IV) in the catalytic domains of dextran-, mutan-, alternan- and reuteransucrase enzymes of lactic acid bacteria (also see (Monchois *et al.*, 1999d)), and amino acid residues with important functional roles, previously identified by mutagenesis, or targeted in this study. GTFA, *Lb. reuteri* 121 (Kralj *et al.*, 2002); GTFBIO, *Lb. reuteri* BioGaia (Kralj *et al.*, 2004a); GTF180 of *Lb. reuteri* 180 (Kralj *et al.*, 2004b); GTFML1 of *Lb. reuteri* ML1 (Kralj *et al.*, 2004b); GTFB, *S. mutans* GS5 (Shiroza *et al.*, 1987); GTFD, *S. mutans* GS5 (Honda *et al.*, 1990); GTFI, *S. downei* MFe28 (Ferretti *et al.*, 1987); GTFS, *S. downei* MFe28 (Gilmore *et al.*, 1990); GTFJ, *Streptococcus salivarius* ATCC 25975 (Giffard *et al.*, 1991); GTFK, *S. salivarius* ATCC 25975 (Giffard *et al.*, 1993); DSRB, *Ln. mesenteroides* NRRL B-1299 (Monchois *et al.*, 1998a); DSRS, *Ln. mesenteroides* NRRL B-512F (Monchois *et al.*, 1997); ASR, *Ln. mesenteroides* NRRL B-1355 (Arguello-Morales *et al.*, 2000); DSRE CD1 and CD2, *Ln. mesenteroides* NRRL-B1299(Bozonnet *et al.*, 2002).*****, identical residue; **:**, highly conserved residue; **.** , conserved residue; ∀, putative catalytic nucleophile (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); ⇓, putative acid/base catalyst (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); ♦, putative residue stabilizing the transition state intermediate (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997); □, putative acceptor substrate binding / glycosyl transfer sites (MacGregor *et al.*, 1996); ‡, residues involved in glucan solubility and structure determination [α-(1→3)/α-(1→6)] as shown separately for different glucansucrases, mutated amino acids shown in bold face and underlined (Shimamura *et al.*, 1994, Remaud-Simeon *et al.*, 2000, Monchois *et al.*, 2000a). The GTFB tryptophan residue shown underlined is important for activity, its mutagenesis resulted in complete loss of enzyme activity (Tsumori *et al.*, 1997). Catalytic amino acids mutated in different studies (Devulapalle *et al.*, 1997, Monchois *et al.*, 1999d, Kralj *et al.*, 2004d) are shown in bold italic type. GTFA amino acid residues targeted in this study are shown in bold type.

members of families GH13 and GH70, however, provide a perfect starting point for investigations of structure/function relationships in GTF enzymes (see below).

Amino acid residues crucial for catalysis in glucansucrases of family GH70 have been identified as Asp1024 (putative catalytic nucleophile, *Lb. reuteri* 121 GTFA numbering), Glu1061 (putative acid/base catalyst) and Asp1133 (putative transition state stabilizer) in GTFI from *Streptococcus downei* Mfe28 and GTFA from *Lb. reuteri* 121 (Fig. 1) (Devulapalle *et al.*, 1997, Kralj *et al.*, 2004d). The equivalent residues, occurring invariably in enzymes of family GH13, are Asp229, Glu257 and Asp328 (*Bacillus circulans* 251 CGTase numbering), respectively (Uitdehaag *et al.*, 1999). In enzymes of both families the catalytic nucleophile (Asp229 and Asp1024, respectively) is involved in formation of the covalent glucosyl-enzyme complexes (Mooser *et al.*, 1991, MacGregor *et al.*, 1996, Uitdehaag *et al.*, 1999, Jensen *et al.*, 2004). This suggests that initial sucrose cleavage proceeds via a similar mechanism in GTF enzymes as in other glycosidases.

Based on a comparison with sugar-binding acceptor subsites in family GH13 enzymes (MacGregor *et al.*, 1996), the locations of two regions putatively involved in acceptor substrate binding in GTF enzymes were identified, C-terminal of the catalytic residues D1024 (GTFA numbering, region II, Fig 1) and E1061 (region III, Fig. 1). A third (putative) acceptor substrate binding region was identified on basis of earlier mutagenesis studies with different GTF enzymes, involving amino acid residue 1138 and 1142 (GTFA, *Lb. reuteri* 121 numbering), located C-terminal of the catalytic residue D1133 (region IV, Fig. 1), determining the solubility of the glucan products and the ratio of [α-(1→3) versus α-(1→6)] glucosidic linkages present (see below) (Shimamura *et al.*, 1994, Remaud-Simeon *et al.*, 2000, Monchois *et al.*, 2000b).

Several additional GTF enzymes recently have been identified in different *Lb. reuteri* strains. This allowed characterization of four highly similar (~70% identity, ~80% similarity) *Lb. reuteri* GTF enzymes that nevertheless synthesize different glucan products (dextran, GTF180 of *Lb. reuteri* 180; mutan, GTFML1 of *Lb. reuteri* ML1; reuteran, GTFA of *Lb. reuteri* 121; reuteran, GTFBIO of *Lb. reuteri* BioGaia) (Kralj *et al.*, 2002, Kralj *et al.*, 2004d, Kralj *et al.*, 2004a, Kralj *et al.*, 2004b). GTFBIO synthesizes a reuteran containing a larger number of α-(1→4) glucosidic linkages (~70%) compared to GTFA reuteran (~45%). Based on the new glucansucrase (and reuteransucrase) sequence information, GTFA amino acid residues putatively responsible for glucosidic linkage specificity located close to the three catalytic residues and putative acceptor substrate binding regions were identified as targets for site-directed mutagenesis experiments (Fig. 1).

The region directly C-terminal of the catalytic Asp1024 contains the conserved amino acids Asp1024-Ala-Val(Pro)-Asp-Asn-Val(Ile)1029 (GTFA numbering). In the second catalytic domain (CD2) of DSRE of *Ln. mesenteroides* NRRL B-1299 (responsible for α-(1→2,6) linkage synthesis) the second Val residue is replaced by an Ile residue (Ile2215)

(Fig. 1). The Pro1026 and Ile1029 combination is present only in GTFA and GTFBIO, strikingly the only glucansucrases that synthesize reuterans [α-(1→4)] polymers) (Kralj *et al.*, 2002, Kralj *et al.*, 2004a). Therefore, these two GTFA residues were mutated (P1026V and I1029V, Fig. 1).

The region following the putative acid/base catalyst E1061 is less conserved (Monchois *et al.*, 1999d, Kralj *et al.*, 2003). GTFA possesses an Ala residue at amino acid position 1066, while an Asn residue is located here in most other glucansucrases (Fig. 1). Therefore, this GTFA residue was mutated (A1066N). Both reuteransucrase (GTFA and GTFBIO) enzymes are highly similar in the three (putative) acceptor substrate binding regions and only differ in two amino acid residues in the region following the acid/base catalyst: Glu1061-Asp-Trp-Asn-His(Ser)-Ala(Ser)-Asp1067 (residues shown in brackets are present in GTFBIO). Thus, residues 1065 and 1066 of GTFA were exchanged by site-directed mutagenesis for the residues present in GTFBIO (H1065S:A1066S, Fig. 1).

The region following D1133 (transition state stabilizer) in both reuteransucrases (GTFA and GTFBIO) differs from the sequence Asp1133-Ser-Glu-Val-Gln-Thr-Val-Ile1140, conserved in many glucansucrases from *Streptococcus*, *Leuconostoc* and *Lactobacillus* species (Fig. 1) (Monchois *et al.*, 1999d, Kralj *et al.*, 2003, Kralj *et al.*, 2004b). In both GTFA (reuteran) and GTFBIO (reuteran), an original tripeptide is found immediately downstream of this catalytic Asp, Asn-Asn-Ser. Also GTF180 (dextran) and GTFML1 (mutan) contain both an original tripeptide at this position, Ser-Asn-Ala and Asn-Gly-Ser, respectively (Kralj *et al.*, 2003, Kralj *et al.*, 2004b). Finally, also alternansucrase (ASR) of *Ln. mesenteroides* NRRL B-1355 (alternan) and CD2 of DSRE of *Ln. mesenteroides* NRRL-B1299 contain both an original tripeptide at this position, Tyr-Asp-Ala and Lys-Gly-Val, respectively (Fig. 1) (Arguello-Morales *et al.*, 2000, Bozonnet *et al.*, 2002).

In different GTF enzymes, mutations in an amino acid residue located five amino acids behind the catalytic Asp1133, T589E in GTFD of *S. mutans* GS5, D567T in GTFB of *S. mutans* GS5 (Shimamura *et al.*, 1994), T667R in DSRS of *Ln. mesenteroides* NRRL B-512F (Remaud-Simeon *et al.*, 2000), and D569X in GTFI of *S. downei* Mfe28 (Monchois *et al.*, 2000b), resulted in a shift in glucan (in)solubility and/or the type of glucosidic bonds [α-(1→3) versus α-(1→6)] synthesized (Fig. 1). Mutation T589E in GTFD of *S. mutans* GS5 lowered the amount of soluble glucan synthesized from 86% to 2% (Shimamura *et al.*, 1994). The reverse shift, from a completely insoluble glucan to more soluble glucan synthesis, was observed when the similar amino acid residue was mutated in GTFB (mutant D567T) of *S. mutans* GS5 (increase in soluble glucan from 0% to 24 %) and in GTFI (mutant D569T) of *S. downei* Mfe28 (Shimamura *et al.*, 1994, Monchois *et al.*, 2000b). Mutation T667R in DSRS of *Ln. mesenteroides* NRRL B-512F resulted in 8% more α-(1→3) linkages in the dextran product (Remaud-Simeon *et al.*, 2000) (Fig. 1). Also mutation D571K in GTFB resulted in increased soluble glucan

synthesis (from 0 to 18%) (Shimamura *et al.*, 1994) (Fig. 1). GTF proteins mutated in the tripeptide immediately following the putative transition state stabilizing residue have not been reported yet. This GTFA region therefore was mutated, substituting the unique triplet Asn-Asn-Ser for the conserved Ser-Glu-Val residues found in many other glucansucrases (N1134S:N1135E:S1136V). Finally, various combinations of the mutations made in the separate acceptor substrate binding regions were constructed and analysed as well.

The effect of the amino acid substitutions on GTFA enzyme activity, glucan and oligosaccharide synthesis, were determined, aiming for a better understanding of the functional roles of these amino acids and regions in glucansucrase enzymes, with emphasis on glucan and oligosaccharide synthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions

Escherichia coli TOP 10 (Invitrogen) was used as host for cloning purposes. Plasmid pBluescript II SK^+ (Stratagene) was used for cloning purposes. Plasmid pET15b (Novagen) was used for expression of the different *gtf* genes in *E. coli* BL21 Star (DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium (Ausubel *et al.*, 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the 100 μg ml^{-1} ampicillin. Agar plates were made by adding 1.5 % agar to the LB medium.

Molecular techniques

General procedures for cloning, *E. coli* transformations, DNA manipulations, and agarose gel electrophoresis were as described (Sambrook *et al.*, 1989). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the enzyme suppliers (New England Biolabs; Roche Biochemicals). Primers were obtained from Eurogentec, Seraing, Belgium. DNA was amplified by PCR on a DNA Thermal Cycler PTC-200 (MJ Research) using *Pwo* DNA polymerase (Roche Biochemicals). Fragments were isolated from agarose gels using a Qiagen gel extraction kit (Qiagen) following the instructions of the supplier.

Construction of plasmids for site-directed mutagenesis experiments

Plasmid pBPE1500, containing the 3'- part of the catalytic core of the *gtfA* gene of *Lb. reuteri* 121 (Kralj *et al.*, 2004d) was used as template for mutagenesis. The QuickChange™ site-directed mutagenesis kit (Stratagene) was used to construct mutants P1026V, I1029V, A1066N, H1065S:A1066S and N1134S:N1135E:S1136V. Constructs

with multiple mutations were made using pBPE1500 containing mutation(s) as template and different primer pairs (Table 1). Successful mutagenesis resulted in appearance of new restriction sites, allowing rapid screening of potential mutants (Table 1). After successful mutagenesis (confirmed by DNA nucleotide sequencing), the pBPE1500 derivative (containing insert with mutation) was digested with *Pst*I and *Eco*RV and ligated in the corresponding sites of pBGTF2 (containing full length GTFA with a C-terminal His-tag) (Kralj *et al.*, 2004d). The resulting plasmid pBGTF2 (containing insert with mutation) was digested with *Nco*I/*Bam*HI and the resulting 5.3 kb fragment was ligated into the corresponding sites of the expression vector pET15b (Novagen) yielding p15GTF2 containing insert with mutation. Plasmid pBGTF2 containing mutation H1065S:A1066S was digested with *Nco*I/*Xho*I and the resulting 4.5 kb fragment was ligated into the corresponding sites of the vector p15GTF2 (Kralj *et al.*, 2004d).

Table 1. Templates and oligonucleotides used for site-directed mutagenesis of *gtfA*. Nucleotides in bold type/italics represent mismatches with the sequence of *gtfA*. Underlined nucleotides represent introduced restriction sites.

Mutation in GTFA protein	Template	Primer pairs (5'→3')	Introduced Restriction site
P1026V	pBPE 1500	GTAGATGCA***GTC***GACAATATTGATGCCG CGGCATCAATATTGTC***GAC***TGCATCTAC	*Sal*I
I1029V	pBPE 1500	GTAGATGCACCGGATAAT***GTC***GACGCC GGCGTC***GAC***ATTATCCGGTGCATCTAC	*Sal*I
P1026V:I1029V	pBPE 1500 P1026V	GTAGATGCACCGGATAAT***GTC***GACGCC GGCGTC***GAC***ATTATCCGGTGCATCTAC	*Sal*I
A1066N	pBPE 1500	GAAGACTGGAATCAT***AA***TGA***C***CCGGAATAC GTATTCCGG***G***TCA***TT***ATGATTCCAGTCTTC	*Nci*I
H1065S:A1066S	pBPE 1500	GAAGACTGGAAT***TC***T***T***C***G***GATCCGGAATACTTT AAAGTATTCCGGATC***C***G***A***A***GA***ATTCCAGTCTTC	*Bam*HI
P1026V:I1029V:A1066N	pBPE 1500 P1026V:I1029V	GAAGACTGGAATCAT***AA***TGA***C***CCGGAATAC GTATTCCGG***G***TCA***TT***ATGATTCCAGTCTTC	*Nci*I
N1134S:N1135E:S1136V	pBPE 1500	CGTTCGGGC***C***CACGATA***G***T***G***A***AGT***TCAAGATCAAATTCAAAATGC GCATTTTGAATTTGATCTTGA***ACT***T***C***A***C***TATCGTG***G***GCCCGAACG	*Apa*I
P1026V:I1029V: N1134S:N1135E:S1136V	pBPE 1500 P1026V:I1029V	CGTTCGGGC***C***CACGATA***G***T***G***A***AGT***TCAAGATCAAATTCAAAATGC GCATTTTGAATTTGATCTTGA***ACT***T***C***A***C***TATCGTG***G***GCCCGAACG	*Apa*I
A1066:N:N1134S:N1135E:S1136V	pBPE 1500 N1134S:N1135E:S1136V	GAAGACTGGAATCAT***AA***TGA***C***CCGGAATAC GTATTCCGG***G***TCA***TT***ATGATTCCAGTCTTC	*Nci*I
P1026V:I1029V: A1066N:N1134S:N1135E:S1136V	pBPE 1500 P1026V:I1029V: N1134S:N1135E:S1136V	GAAGACTGGAATCAT***AA***TGA***C***CCGGAATAC GTATTCCGG***G***TCA***TT***ATGATTCCAGTCTTC	*Nci*I

Purification of GTFA (mutant) proteins

Wild type and mutant GTFA derivatives were expressed in *E. coli*, produced, extracted and purified as described previously (Kralj *et al.*, 2004d). The degree of purity was determined by SDS-PAGE (Laemmli, 1970). Protein concentrations were determined using the Bradford method using the Bio-Rad reagent and bovine serum albumin as a standard (Bio-Rad).

Enzyme activity assays

The various reuteransucrase activities were determined as initial rates by measuring glucose and fructose release (enzymatically) from sucrose conversion (van Geel-Schutten *et al.*, 1999, Kralj *et al.*, 2004d). The amount of fructose released (V_F) corresponds to total enzyme activity (initial formation of leucrose and other sucrose isomers was negligible, see results and data not shown). The amount of free glucose (V_G) represents the hydrolytic activity of the enzyme. The amount of fructose minus the amount of free glucose reflects the transferase activity (V_F-V_G). Unless indicated otherwise, reactions were performed at 50 °C in 25 mM NaAc buffer, pH 4.7, containing 1 mM $CaCl_2$ and 30 nM purified (mutant) reuteransucrase enzyme. One unit of enzyme activity is defined as the release of 1 µmol of monosaccharide per min. Kinetic parameters. Kinetic assays were performed using fifteen different sucrose concentrations ranging from 0.25 to 100 mM. Over a 6 min incubation period, samples of 25 µl were withdrawn every minute and inactivated with 2.5 µl 1 M NaOH. Curve fitting of the data was performed with the "SigmaPlot" program (version 8.0) using either the Michaelis-Menten formula [$y = (a \times x) / (b + x)$], or the same formula with a substrate inhibition constant [$y = (a \times x) / (b + x + (x^2/c))$]. In these equations y is the specific activity ($U.mg^{-1}$), x is the substrate concentration (mM sucrose), a is the maximal reaction rate, *V*max ($U.mg^{-1}$), b is the affinity constant for the substrate (*K*m, mM sucrose), and c is the substrate inhibition constant (*K*i, mM sucrose).

***In vitro* glucan production by (mutant) GTFA enzymes and glucan structure analysis**

(i) Polymer production. Purified (mutant) enzyme preparations were incubated with 146 mM sucrose, using the conditions described above under enzyme activity assays. Glucans produced were isolated by precipitation with ethanol as described previously (van Geel-Schutten *et al.*, 1999).

(ii) Methylation analysis. Polysaccharides were permethylated using methyl iodide and dimsyl sodium ($CH_3SOCH_2^-$-Na^+) in DMSO at room temperature (Hakomori, 1964). After hydrolysis with 2 M trifluoroacetic acid (1 h, 125 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ (Harris *et al.*, 1984). Mixtures of partially methylated alditol acetates obtained were analyzed by GLC on a CP Sil 5 CB column (25 m × 0.53 mm; Chrompack) and by GLC-mass spectrometry (MS) on a RTX 5 Sil MS (30 m × 0.25 mm; Restek) column (Chaplin, 1982, Jansson *et al.*, 1976).

(iii) ^{1}H-NMR spectroscopy. Prior to NMR spectroscopy samples were dissolved in 99.96 atom % D_2O (Isotec). One-dimensional ^{1}H-NMR spectra were recorded on a 600 MHz Bruker AVANCE NMR spectrometer at a probe temperature of 353K. The HOD signal was suppressed by applying a pressat sequence. Chemical shifts are expressed in ppm by reference to external acetone (δ=2.225). Proton spectra were recorded in 64K data sets,

with a spectral width of 8,000 Hz. Resolution enhancement of the spectra was performed with a Lorentzian-to-Gaussian transformation; when necessary, a fifth-order polynomial baseline correction was performed.

Product spectrum from sucrose

After complete depletion of sucrose (100 mM, 60 h at 35 °C) by 30 nM GTFA (mutant) enzyme, the concentrations of fructose, glucose, isomaltose and leucrose in the reaction medium were determined using Anion-Exchange chromatography (Dionex) as described previously (Kralj *et al.*, 2004d). The amounts of free fructose released from sucrose (97.9 %) and leucrose synthesized from sucrose (2.1 %) correspond to 100 % sucrose converted. Subtracting the free glucose released from sucrose (22.7 %; due to hydrolysis) and isomaltose (1.6 %) synthesized from sucrose from the free fructose (97.9 %) released from sucrose, allowed calculation of the yield of reuteran synthesis (73.7 %) from sucrose (data of wild type GTFA used here for clarification, see Table 3).

Synthesis and characterization of oligosaccharides

(i) Synthesis and detection. Oligosaccharide synthesis was investigated using sucrose (100 mM) and the acceptor substrates maltose and isomaltose (100 mM). Products were analyzed with Anion-Exchange chromatography (Dionex) as described previously (Kralj *et al.*, 2004d). No standards were available for α-(1→6)-panose, isopanose and α-(1→6)-isopanose. Therefore, the calibration curve for panose, representing the most closely related oligosaccharide available to us, was used to estimate the approximate concentrations of these three compounds. Total and individual oligosaccharide yields were calculated from the amount of acceptor substrate converted into total and individual oligosaccharides, expressed as a percentage of the total amount of acceptor substrate initially present in the incubation. The Dionex analysis protocol used allowed recovery of 85-110% of the (iso)maltose initially present in the incubation, as remaining (iso)maltose and/or as oligosaccharide products formed. The lower percentages maybe due to synthesis of unknown products from (iso)maltose. Both the lower and higher percentages maybe due to use of the panose calibration curve to determine concentrations of the three other compounds (see above).

(ii) Purification. To determine the degree of polymerization (DP) and to purify the oligosaccharides, a BC-200 Ca^{2+} column (at 85 °C; 300 by 7.8 mm; Benson Polymeric) eluted with water (0.2 ml min^{-1}) was used (linear maltooligosaccharides were used to calibrate the system). Detection was done using a model 830-RI refractive index detector at 40 °C (Jasco).

(iii) Characterization. The separate purified oligosaccharides were subjected to enzyme degradation using dextranase from *Penicillium* sp (EC 3.2.1.11, Sigma), which hydrolyzes only α-(1→6) glucosidic bonds (Bourne *et al.*, 1963, Taylor *et al.*, 1995), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, Sigma), which was shown to hydrolyze α-(1→4), α-(1→3) and α-(1→6) linkages at decreasing rates, respectively, to produce glucose from the non-reducing end of linear oligosaccharides (Pazur & Ando, 1960, Pazur & Klepppe, 1962), and α-glucosidase from *Bacillus stearothermophilus* (EC 3.1.2.20 Megazyme, Ireland), which hydrolyzes terminal, α-(1→4) linkages from the non-reducing end of oligosaccharides to produce glucose (Mala *et al.*, 1999). Oligosaccharides (1 $g.l^{-1}$) were incubated with 0.1 U ml^{-1} amyloglucosidase, 66 U ml^{-1} dextranase or 66 U ml^{-1} α-glucosidase. After 30 min, 2 h and 18 h of incubation, samples were withdrawn and products formed (in time) were analyzed by Anion-Exchange chromatography (see above). One endodextranase unit is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmole isomaltose from dextran min^{-1} at 37 °C and pH 6.0. One amyloglucosidase unit is defined as the amount enzyme that hydrolyzes 1 mg of maltose per 3 min at 55 °C and pH 4.5. One α-glucosidase unit is defined as the amount of enzyme that hydrolyzes 1 μmole ρ-nitrophenol-α-glucoside min^{-1} at 40 °C and pH 6.5.

RESULTS

Influence of mutations on kinetic parameters

Mutants in conserved region I, near the catalytic D1024 residue (P1026V, I1029V and P1026V:I1029V), displayed 1.5 to 2 fold higher activities than GTFA wild type in all three reactions catalyzed. Affinities for sucrose in the hydrolysis and transferase reactions were slightly lower (Table 2A).

Mutant A1066N, in the vicinity of the catalytic E1061 residue, displayed similar kinetic parameters as wild type GTFA (Table 2A). The double mutant H1065S:A1066S lacked transferase activity (initial rates; Table 2A). The hydrolytic activity of this mutant was 2 fold lower than in wild type, whereas the affinity for sucrose in the hydrolysis reaction was 2 fold higher compared to wild type.

The combination of the P1026V:I1029V and A1066N mutations resulted in higher activities in all reactions, whereas affinities for the substrate sucrose were comparable to wild type levels in all reactions (Table 2A).

Both triple N1134S:N1135E:S1136V and quadruple A1066N:N1134S:N1135E:S1136V mutants showed no transferase activity at low sucrose concentrations (initial rates); the affinity of both mutants for the substrate sucrose in the transferase reaction was extremely low. The affinity for sucrose for total activity was also strongly decreased

(Table 2A). The same phenomena were observed for the other two mutants containing the N1134S:N1135E:S1136V mutation in combination with mutations in the other (putative) acceptor substrate binding region(s). Normal Michelis-Menten kinetics was observed for the hydrolysis reaction of the four mutants containing mutation N1134S:N1135E:S1136V, whereas wild type and the other mutants showed substrate inhibition for the hydrolysis reaction (Table 2A).

The *V*max for total activity (V_F) in the reaction with sucrose and maltose as acceptor reaction substrate had increased for most mutants. Mutant P1026V:I1029V:A1066N showed a similar activity as wild type; mutant H1065S:A1066S displayed a three fold reduced activity (Table 2B).

Table 2. Kinetic parameters of GTFA wild type and site-directed mutants derived: (**A**) Kinetic parameters were determined using 15 different concentrations of sucrose (0.25-100 mM); (**B**) Total activity (initial rates) determined using 50 mM sucrose and 100 mM maltose. * The absence of transferase activity at low substrate concentrations resulted in a high standard error with curve fitting. Data obtained were fitted using the Michaelis-Menten equation (with or without (#) substrate inhibition). ND: no transferase activity (initial rate) could be detected. *f* Total activity for mutant H1065A:A1066S was fitted using the Michaelis-Menten equation with substrate inhibition: *K*i 64.1 ± 16.9 mM.

A	Total Activity		Transferase Activity		Hydrolysis Activity			B Acceptor Reaction
Enzyme	$V\text{max}^F$ ($U.mg^{-1}$)	$K\text{m}^F$ (mM)	$V\text{max}^{F\text{-}G}$ ($U.mg^{-1}$)	$K\text{m}^{F\text{-}G}$ (mM)	$V\text{max}^G$ ($U.mg^{-1}$)	$K\text{m}^G$ (mM)	*K*i (mM)	$V\text{max}^F$ ($U.mg^{-1}$)
Wild type	28.4 ± 1.0	0.9 ± 0.2	11.9 ± 0.8	4.8 ± 1.1	24.3 ± 1.0	1.0 ± 0.1	106.9 ± 16.8	97.7 ± 0.9
P1026V	55.2 ± 2.2	1.8 ± 0.3	24.4 ± 1.2	8.3 ± 1.4	46.0 ± 4.3	1.6 ± 0.4	144.9 ± 54.6	130.0 ± 5.5
I1029V	38.1 ± 1.2	1.4 ± 0.2	19.5 ± 1.9	8.5 ± 1.4	29.9 ± 2.0	1.3 ± 0.2	105.6 ± 25.0	122.0 ± 8.5
P1026V:I1029V	45.0 ± 3.2	1.2 ± 0.3	17.0 ± 1.2	9.0 ± 1.8	44.1 ± 5.2	1.4 ± 0.4	76.2 ± 25.8	110.5 ± 3.4
A1066N	29.9 ± 1.3	1.1 ± 0.2	14.5 ± 0.9	3.3 ± 0.7	22.6 ± 1.0	1.1 ± 0.1	103.4 ± 16.6	114.6 ± 0.2
H1065S:A1066S	12.1 ± 1.1*f*	1.2 ± 0.3	ND	ND	10.6 ± 0.6	1.1 ± 0.2	56.6 ± 9.9	33.4 ± 2.1
P1026V:I1029V:A1066N	39.0 ± 2.0	1.1 ± 0.2	16.1 ± 1.6	5.1 ± 1.7	34.5 ± 2.3	1.2 ± 0.2	95.7 ± 22.1	97.3 ± 1.1
N1134S:N1135E:S1136V	27.6 ± 1.6	8.5 ± 1.6	102.6 ±71.6*	540.9 ± 432.8*	13.9 ± 0.2	2.6 ± 0.1	-#	110.2 ± 1.7
P1026V:I1029V: N1134S:N1135E:S1136V	45.7 ± 1.4	4.8 ± 0.5	29.4 ± 2.4	36.7 ± 7.2	26.1 ± 0.5	2.3 ± 0.2	-#	120.1 ± 2.1
A1066N: N1134S:N1135E:S1136V	19.8 ± 1.0	7.9 ± 1.3	35.8 ±13.9*	224.9 ±116.8*	10.2 ± 0.3	2.8 ± 0.3	-#	125.2 ± 2.5
P1026V:I1029V:A1066N: N1134S:N1135E:S1136V	31.2 ± 0.9	4.1 ± 0.4	17.2 ± 1.4	33.1 ± 6.5	19.6 ± 0.2	2.2 ± 0.1	-#	102.5 ± 1.4

Influence of mutations on oligosaccharide synthesis

(i) Product spectrum from sucrose

The single mutant P1026V, the double mutant P1026V:I1029V and the triple mutant P1026V:I1029V:A1066N showed 3 to 4 fold increased levels of isomaltose production

(and a slight decrease in reuteran synthesis) with sucrose alone or with sucrose and fructose as acceptor reaction substrate (Table 3 and data not shown) compared to wild type. Compared to wild type GTFA, these three mutants showed up to 2 fold higher leucrose yields when incubated with sucrose alone, or with sucrose and fructose as acceptor reaction substrate (~10 mM leucrose synthesized) (Table 3 and data not shown). All other mutants showed similar product spectra as wild type GTFA upon incubation with sucrose (Table 3).

The triple mutant N1134S:N1135E:S1136V, incubated with 100 mM sucrose for 60 h, did not consume all sucrose. Only prolonged incubation resulted in complete consumption of sucrose. The other three mutants containing this triple amino acid mutation displayed the same properties.

Table 3. Product spectra of GTFA wild type and derived site-directed mutants after 60 h of incubation. Percentages indicate the relative conversion of sucrose into reuteran, oligosaccharides (leucrose and isomaltose) and glucose (hydrolysis). The 100% value is equivalent to the total amount of sucrose consumed after 60 h of incubation, in most cases resulting in sucrose depletion. * Sucrose consumed for 20-70% after 60 h of incubation. *f* data from (Kralj *et al.*, 2004d).

Enzyme	Reuteran (%)	Leucrose (%)	Isomaltose (%)	Glucose (%)
Wild type*f*	73.7 ± 3.6	2.1 ± 0.1	1.6 ± 0.2	22.7 ± 3.2
P1026V	66.1 ± 2.9	3.8 ± 0.4	6.2 ± 0.5	23.9 ± 2.0
I1029V	74.1 ± 3.2	2.3 ± 0.3	1.7 ± 0.3	21.9 ± 2.6
P1026V:I1029V	65.0 ± 4.0	4.0 ± 0.1	6.2 ± 0.7	24.8 ± 3.2
A1066N	76.3 ± 2.7	1.9 ± 0.1	1.4 ± 0.2	20.4 ± 2.5
H1065S:A1066S	73.4 ± 0.4	1.7 ± 0.1	1.4 ± 0.1	23.5 ± 0.2
P1026V:I1029V:A1066N	67.3 ± 2.9	3.6 ± 0.1	5.5 ± 0.3	23.5 ± 2.5
N1134S:N1135E:S1136V	74.6 ± 1.2*	1.9 ± 0.3	0.6 ± 0.1	23.0 ± 1.3
P1026V:I1029V:N1134S:N1135E:S1136V	76.5 ± 0.1*	3.0 ± 0.6	1.0 ± 0.1	19.5 ± 0.6
A1066N:N1134S:N1135E:S1136V	69.1 ± 1.7*	0.7 ± 0.2	0.5 ± 0.2	29.7 ± 2.4
P1026V:I1029V:A1066N:N1134S:N1135E:S1136V	72.9 ± 1.0*	2.1 ± 0.4	1.1 ± 0.1	23.9 ± 0.5

(ii) Oligosaccharide synthesis with maltose as acceptor substrate. When sucrose and maltose were both present in the assay mixtures, GTFA synthesized mostly panose and some maltotriose (Table 4) (Kralj *et al.*, 2004d). All GTFA derived enzyme variants (including the four enzymes containing the triple amino acid mutation N1134S:N1135E:S1136V) were able to completely consume sucrose within 60 h. Wild type GTFA and all mutants derived produced similar percentages of oligosaccharides

(~60%) from sucrose and maltose as acceptor reaction substrate (Table 4). Interestingly, the four enzymes with the N1134S:N1135E:S1136V mutations synthesized an oligosaccharide of DP4 from sucrose and maltose as acceptor reaction substrate (Fig. 2). The structure of this oligosaccharide was identified by enzymatic degradation as α-D-gucopyranosyl-(1→6)-α-D-gucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-D-glucose; α-(1→6)-panose (data not shown). Dextranase degraded the DP4 oligosaccharide into maltose and isomaltose. Amyloglucosidase and alpha glucosidase cleaved the oligosaccharide slowly to glucose and panose (α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose). In contrast, isopanose (α-D-glucopyranosyl-(1→4-α-D-glucopyranosyl-(1→6)]-D-glucose) was degraded very fast by the latter two enzymes (Kralj *et al.*, 2004a). Wild type GTFA and the other mutants did not produce α-(1→6)-panose at all. The four enzymes containing the triple amino acid mutation

Table 4. Product spectra of GTFA and site-directed mutants derived after 60 h of incubation with 100 mM sucrose and 100 mM maltose. # The calibration curve of panose was used to calculate 1,6-panose; [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-[α-D-glucopyranosyl-(1→4)]-D-glucose] concentrations. The total and individual oligosaccharide yields indicate the amount of maltose consumed as a percentage of the total amount of maltose initially present in the incubation. *f* data from (Kralj *et al.*, 2004d). (-), not detectable.

Enzyme	**Oligosaccharide yield (%)**	**Panose (%)**	**Maltotriose (%)**	**1,6-Panose (%)#**
Wild type*f*	59.6 ± 3.9	54.5 ± 4.2	5.1 ± 0.4	-
P1026V	55.1 ± 7.8	51.7 ± 9.6	3.3 ± 1.7	-
I1029V	60.8 ± 4.1	56.9 ± 1.7	3.9 ± 0.3	-
P1026V:I1029V	59.5 ± 3.0	55.9 ± 5.1	3.5 ± 0.5	-
A1066N	62.6 ± 8.1	55.8 ± 8.1	3.9 ± 0.3	-
H1065S:A1066S	63.2 ± 1.1	57.8 ± 1.0	5.3 ± 0.1	-
P1026V:I1029V:A1066N	62.3 ± 2.5	60.4 ± 2.6	1.9 ± 0.1	-
N1134S:N1135E:S1136V	64.8 ± 4.7	40.5 ± 1.3	2.1 ± 0.1	22.1 ± 3.3
P1026V:I1029V:N1134S:N1135E:S1136V	60.7 ± 1.3	34.8 ± 0.2	1.3 ± 0.1	24.7 ± 1.0
A1066N:N1134S:N1135E:S1136V	68.9 ± 4.7	45.1 ± 8.6	2.0 ± 0.2	21.8 ± 2.2
P1026V:I1029V:A1066N:N113 4S:N1135E:S1136V	63.4 ± 0.1	37.2 ± 0.1	1.3 ± 0.1	24.9 ± 0.9

N1134S:N1135E:S1136V showed up to 1.5 fold decreased level of panose synthesis and synthesized also lower amounts of maltotriose compared to wild type and the other mutants.

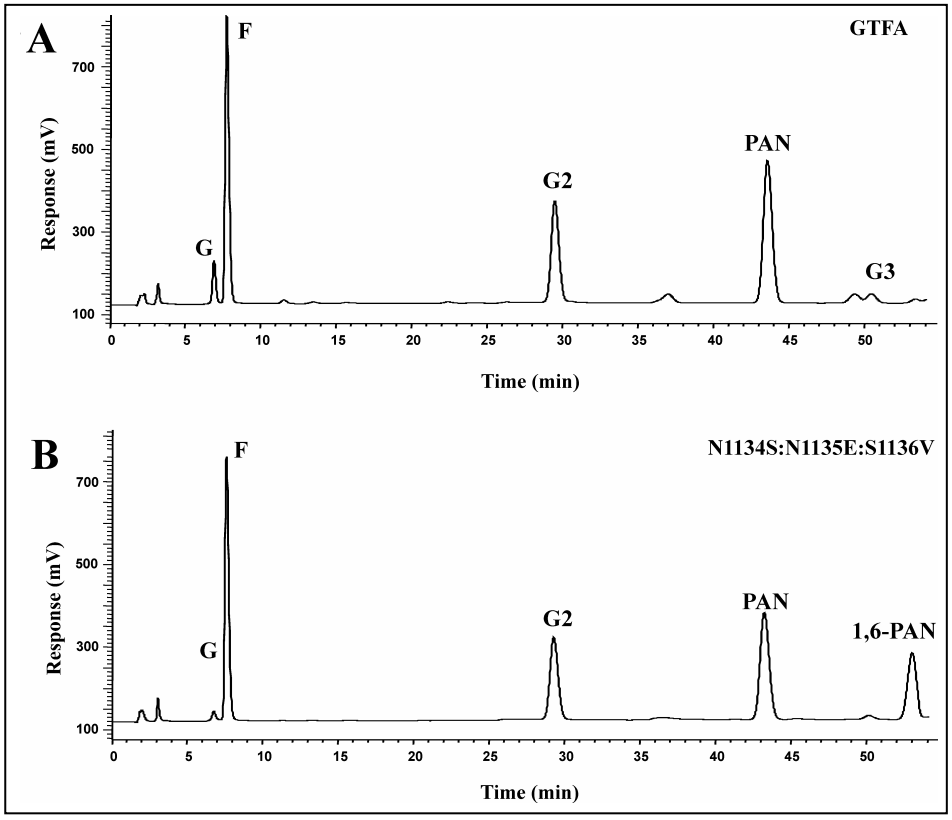

Figure 2. Anion-Exchange (Dionex) analysis of (mutant) GTFA acceptor reaction products produced from sucrose plus maltose. **A**) Products formed upon incubation of 30 nM GTFA enzyme with 100 mM sucrose and 100 mM maltose for 60 h; **B**) Products formed upon incubation of 30 nM GTFA mutant N1134S:N1135E:S1136V enzyme with 100 mM sucrose and 100 mM maltose for 60 h. (F = fructose, G = glucose, G2 = maltose, G3 = maltotriose, PAN = panose, 1,6-PAN =[α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-[α-D-glucopyranosyl-(1→4)]-D-glucose]

(iii) Oligosaccharide synthesis with isomaltose as acceptor substrate. When sucrose and isomaltose were both present in the assay mixtures, GTFA synthesized predominantly isopanose and α-(1→6)-isopanose (Table 5; Fig. 3A) (Kralj *et al.*, 2004d, Kralj *et al.*, 2004a). The three GTFA enzyme variants with the P1026V mutation showed up to five fold increased synthesis of isomaltotriose. These three mutants showed also slightly increased isomaltotetraose synthesis. Oligosaccharide yields from sucrose and isomaltose increased drastically (2 fold) for all four GTFA derived enzyme variants with the N1134S:N1135E:S1136V mutation. These four mutant enzymes also showed a 10 fold

Table 5. Product spectra of GTFA and mutants derived after 60 h of incubation with 100 mM sucrose and 100 mM isomaltose. # The calibration curve of panose was used to calculate isopanose and 1,6-isopanose [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose] concentrations. The total and individual oligosaccharide yields indicate the amount of isomaltose consumed as a percentage of the total amount of isomaltose initially present in the incubation. (-), not detectable.

Enzyme	Oligosaccharide yield (%)	Isopanose (%)#	1,6-isopanose (%)#	Isomalto-triose (%)	Isomalto-tetraose (%)	Isomalto-pentaose (%)	Isomalto-hexaose (%)
Wild type	28.3 ± 2.1	12.2 ± 3.2	11.6 ± 0.8	2.8 ± 0.5	1.6 ± 0.1	-	-
P1026V	29.9 ± 1.8	5.6 ± 0.5	8.9 ± 0.2	12.8 ± 0.5	2.6 ± 0.2	-	-
I1029V	27.3 ± 2.3	8.5 ± 1.7	13.2 ± 0.5	3.9 ± 0.3	1.6 ± 0.1	-	-
P1026V:I1029V	32.0 ± 3.6	6.2 ± 1.3	8.6 ± 0.3	14.2 ± 3.3	2.9 ± 0.6	-	-
A1066N	28.7 ± 0.9	11.6 ± 0.8	11.9 ± 0.1	3.5 ± 0.1	1.7 ± 0.1	-	-
H1065S:A1066S	24.9 ± 2.9	8.6 ± 0.8	11.6 ± 1.6	3.1 ± 0.4	1.6 ± 0.2	-	-
P1026V:I1029V:A1066N	32.6 ± 7.7	6.0 ± 1.4	9.6 ± 2.2	14.1 ± 3.4	2.9 ± 0.7	-	-
N1134S:N1135E:S1136V	61.0 ± 3.4	8.9 ± 0.5	-	27.1 ± 1.4	20.4 ± 1.2	4.6 ± 0.3	-
P1026V:I1029V:N1134S:N1135E:S1136V	61.3 ± 1.2	1.5 ± 0.1	-	26.3 ± 0.4	23.5 ± 0.6	7.2 ± 0.2	2.8 ± 0.1
A1066N:N1134S:N1135E:S1136V	57.3 ± 2.8	7.7 ± 0.3	-	26.5 ± 0.5	19.1 ± 1.4	4.0 ± 0.7	-
P1026V:I1029V:A1066N:N1134S:N1135E:S1136V	59.6 ± 0.5	1.4 ± 0.1	-	26.3 ± 0.3	22.9 ± 0.2	6.7 ± 0.1	2.2 ± 0.1

increased production of isomaltotriose and isomaltotetraose compared to wild type (Table 5, Figs. 3B and C) and no synthesis of α-(1→6)-isopanose. Instead, isomaltopentaose was produced in low amounts. Both mutants N1134S:N1135E:S1136V and A1066N: N1134S:N1135E:S1136V also synthesized minor amounts of an unknown product eluting after ~44 min, which was not synthesized by the quintuple and sextuple mutant enzymes. (Figs. 3B and C). Combination of N1134S:N1135E:S1136V with P1026V:I1029V or P1026V:I1029V:A1066N resulted in a slightly higher production of isomaltotetraose and isomaltopentaose. Only these two mutant enzymes also synthesized (low amounts of) isomaltohexaose. Interestingly, both mutants showed strongly reduced levels of isopanose synthesis, whereas α-(1→6)-isopanose was not produced at all (Table 5; Fig. 3C).

Influence of mutations on glucan synthesis and glucosidic bond specificity

The mutations did not affect conversion of sucrose into glucan polymer during 60 h incubation (Table 3). Methylation and ^{1}H-NMR analysis (Table 6 and Fig. 4) of the polymers produced by the mutant enzymes, showed that mutation P1026V resulted in the production of a polymer with a slightly elevated level of α-(1→6) linkages and a decrease in the amount of α-(1→4) linkages. Mutations I1029V and A1066N did not change the

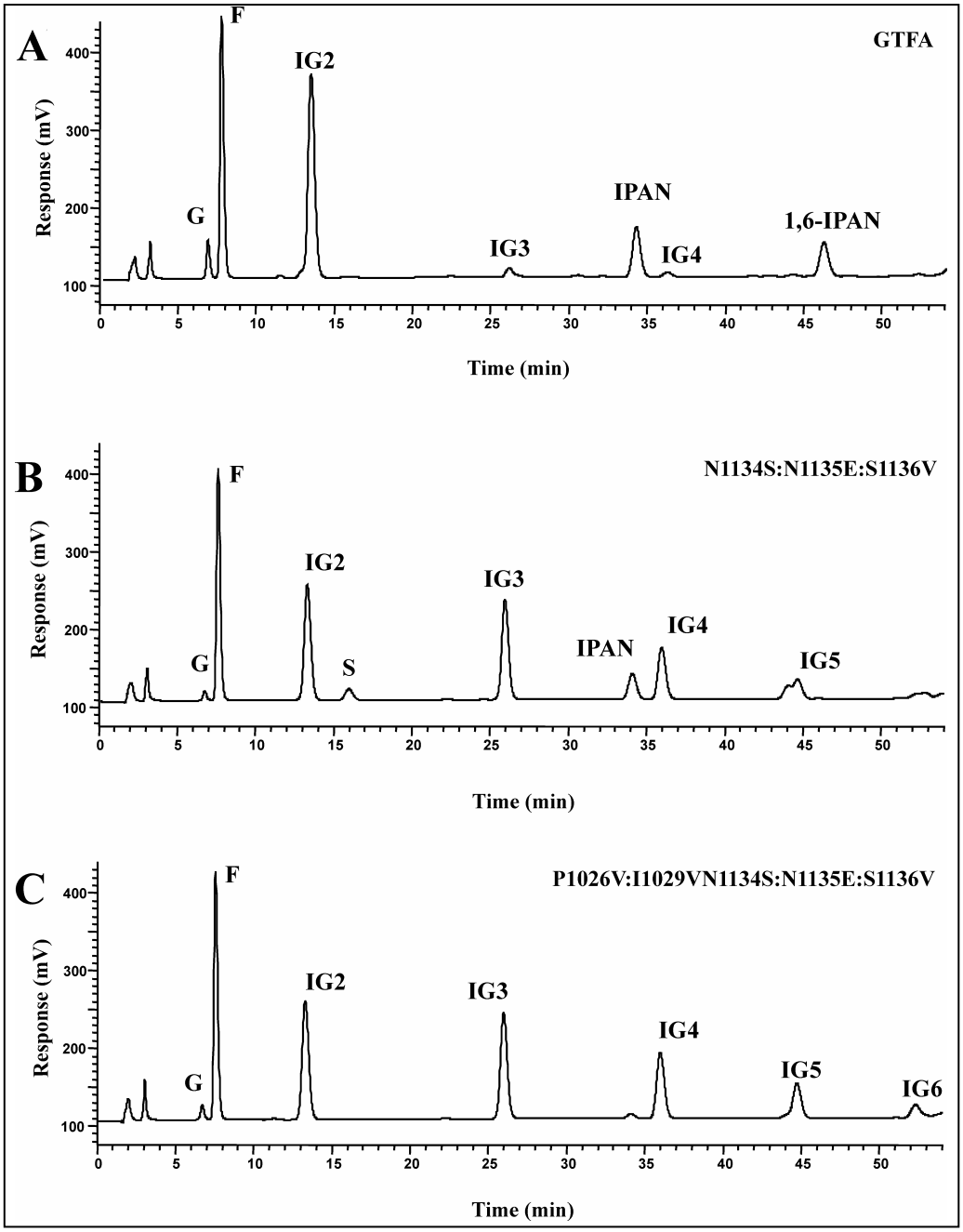

Figure 3. Anion-Exchange (Dionex) analysis of (mutant) GTFA acceptor reaction products produced from sucrose plus isomaltose. **A**) Products formed upon incubation of 30 nM GTFA enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h; **B**) Products formed upon incubation of 30 nM GTFA mutant N1134S:N1135E:S1136V enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h; **C**) Products formed upon incubation of 30 nM GTFA mutant P1026V:I1029V:N1134S:N1135E:S1136V enzyme with 100 mM sucrose and 100 mM isomaltose. (F = fructose, G = glucose, S = sucrose, IG2 = isomaltose, IG3 = isomaltotriose, IG4 = isomaltotetraose, IG5 = isomaltopentaose, IG6 = isomaltohexaose, IPAN = isopanose, 1,6-IPAN = [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose]

nature of the glucan produced (Table 6). The glucan polymers synthesized by the double mutant P1026V:I1029V and the triple mutant P1026V:I1029V:A1066N showed similar distribution of glucosidic linkages inside their polymers as the glucan polymer synthesized by the single mutant P1026V.

Both mutants N1134S:N1135E:S1136V and A1066N:N1134S:N1135E:S1136V showed a drastic increase of α-(1→6) linkages (~40%) and a decrease of α-(1→4) linkages (~40%) in their polymers compared to wild type (Table 6 and Fig. 4). Combination of N1134S:N1135E:S1136V with P1026V:I1029V or P1026V:I1029V:A1066N resulted in an even further increased production of α-(1→6) linkages and a decrease of α-(1→4) linkage synthesis (as shown by methylation and ^{1}H-NMR analysis), yielding a polymer containing very low amounts of α-(1→4) linkages (Table 6 and Fig. 4).

Table 6. Methylation and 600 MHz ^{1}H-NMR analysis of the glucans produced by purified GTFA wild type and derived site-directed mutants. **A**) Methylation analysis. **B**) NMR analysis. The resolution with NMR was too low to trace the terminal and (1→4,6) linked residues as indicated with methylation analysis. Displayed are the anomeric signals at 5.0 ppm (α-(1→6) linkages) and 5.3 ppm (α-(1→4) linkages). ND = not determined.

	A	Methylation (%)			B	Chemical shift (%)	
Type of glucosyl units / Enzyme	Terminal	α-(1→4)	α-(1→6)	α-(1→4,6)		α-(1→4)	α-(1→6)
Wild type	9	46	34	12		57	43
P1026V	10	42	40	8		53	47
I1029V	7	47	35	11		58	42
P1026V:I1029V	7	41	40	12		53	47
A1066N	7	46	36	16		ND	
H1065S:A1066S	10	45	29	16		ND	
P1026V:I1029V:A1066N	6	44	39	10		ND	
N1134S:N1135E:S1136V	7	11	76	6		16	84
P1026V:I1029V:N1134S:N1135E:S1136V	6	2	82	9		8	92
A1066N:N1134S:N1135E:S1136V	6	13	74	7		ND	
P1026V:I1029V:A1066N:N1134S:N1135E:S1136V	6	4	88	3		7	93

DISCUSSION

The different glucansucrase enzymes (GH70) are highly similar in their catalytic cores. Nevertheless, they synthesize a diverse range of linear and branched α-glucans (dextran

[α-(1→6)], mutan [α-(1→3)], alternan [α-(1→3)/α-(1→6)] and oligosaccharides from sucrose (Monchois *et al.*, 1999d). The large diversity in glucan and oligosaccharide products synthesized by different GTF enzymes raised questions about structural features and specific amino acid residues involved in determining the glucosidic linkage specificity. The aim of this study was to identify amino acid residues that determine the linkage specificity in the poly- and oligosaccharide products synthesized by GTFA of *Lb. reuteri* 121.

For amylosucrase and CGTase (family GH13) the different sugar-binding acceptor subsites have been mapped out based on 3D structural information, according to the definition of (Davies *et al.*, 1997). Since GTF enzymes of family GH70 and members of family GH13 have similar structural features (MacGregor *et al.*, 1996, Devulapalle *et al.*, 1997) we postulate that members of both enzyme families have a similar acceptor subsite organisation.

Mutations in conserved region II

GTFA derivatives containing mutation P1026V showed a clear change in oligosaccharide and glucan products, with an increase of α-(1→6) and a decrease of α-(1→4) glucosidic linkages (Tables 3-6). Mutations in an amino acid residue immediately C-terminal of I1029 of GTFA, D457N in GTFB and N471D in GTFD of *Streptococcus mutans* GS5, resulted in an increase of soluble glucan synthesis (from 0 to 37 %) and insoluble glucan synthesis (from 14 to 38%), respectively (Fig. 1). The linkage type and degree of branching of the glucans synthesized by these GTFB and GTFD site-directed mutants have not been reported; conceivably changes in either may have an effect on glucan solubility (Shimamura *et al.*, 1994).

In CGTase the corresponding region constitutes part of the sugar-binding acceptor subsites +1 and +2 (residues Asp^{229} (-1)-Ala- (+1)-Val-Lys-(+2)-His^{233} (+1), in *B. circulans* 251 CGTase), (Leemhuis *et al.*, 2004), responsible for the stereospecific positioning of the molecule accepting the glucosyl unit (Knegtel *et al.*, 1995). The structure of this acceptor site also determines the type of glucosidic bond formed (Uitdehaag *et al.*, 1999). In neopullulanase from *Bacillus stearothermophilus* this region is also important in specificity towards α-(1→4) or α-(1→6) glucosidic linkages (Kuriki *et al.*, 1991).

The postulation made by (MacGregor *et al.*, 1996), that conserved region II in GH13 also is present at the similar position in GH70, is confirmed by our mutant data showing its importance in acceptor binding and glucosyl transfer. The GTFA (Fig. 1) and CGTase (see above) sequences in conserved region II are strongly different, leaving questions about the precise functioning of amino acids in this region in family GH70 members.

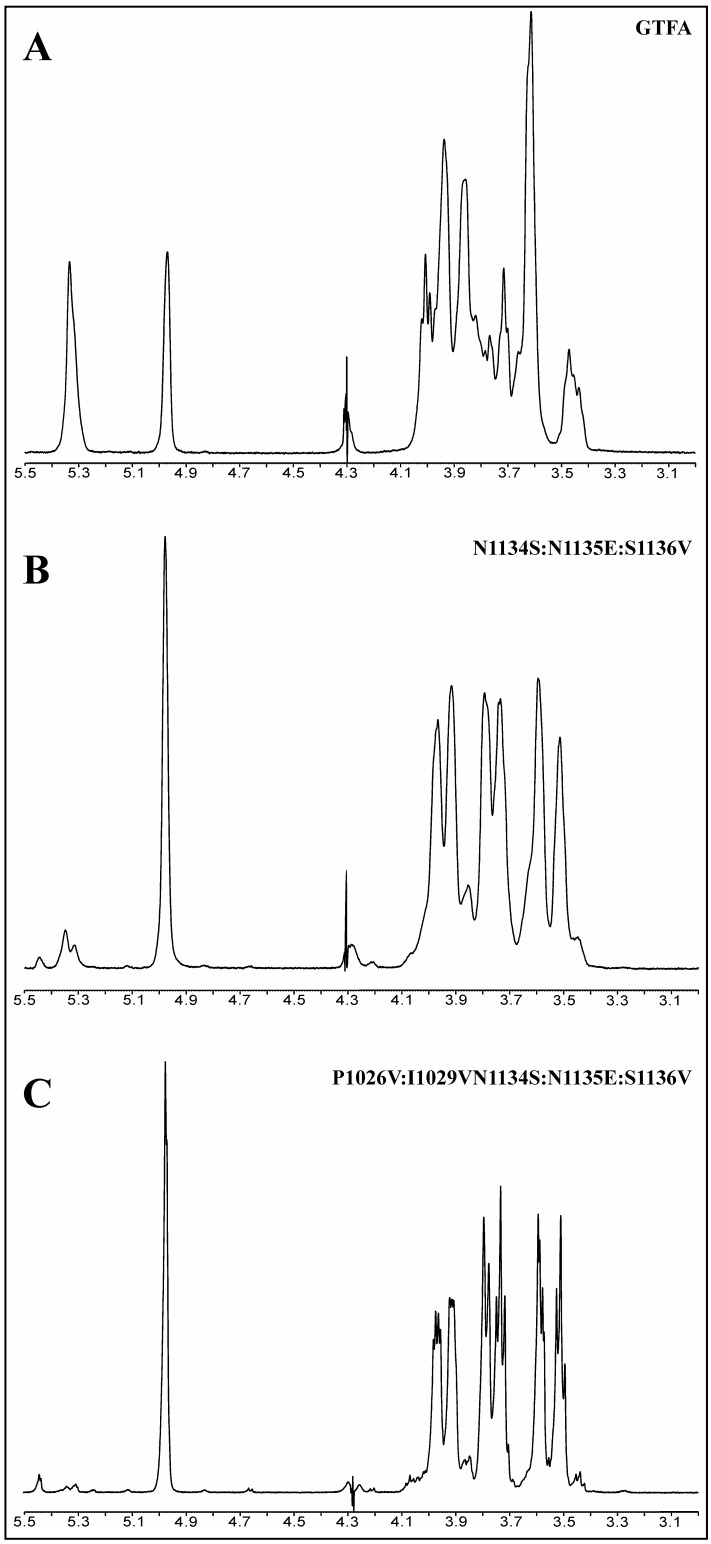

Figure 4. 600-MHz ^{1}H-NMR spectra of the glucans produced by (**A**) purified recombinant GTFA protein, (**B**) GTFA mutant protein N1134S:N1135E:S1136V, and by (**C**) GTFA mutant protein P1026V:I1029V:N1134S:N1135E:S1136V, recorded in D_2O at 80°C. Chemical shifts are given in parts per million relative to the signal of internal acetone (δ=2.225).

Mutations in conserved region III

GTFA mutations A1066N and H1065S:A1066S (making it more similar to GTFBIO) did not result in changes in glucosidic linkages synthesized. However, the double mutant showed a drastic change in enzyme activity: overall activity was lower than in wild type GTFA and no transferase activity could be measured (initial rates) (Table 2). We also failed to detect transferase activity (initial rates) with GTFBIO (Kralj *et al.*, 2004a). Thus, the reaction specificity of this GTFA double mutant has clearly changed, with hydrolysis being the dominant activity, similar to the situation in GTFBIO (initial rates) (Kralj *et al.*, 2004a).

Mutation W491G in GTFB of *S. mutans* GS5, equivalent to F259 in *B. circulans* 251 CGTase, resulted in complete loss of enzyme activity (for unknown reasons) (Tsumori *et al.*, 1997). This Trp residue is almost completely conserved in GTF enzymes (Fig. 1). Mutations in the region following the catalytic glutamate in the CGTase enzymes of *B. circulans* 251 and *Thermoanaerobacterium thermosulfurigenes* strain EM1 (F259X subsite +2 and E264A subsite +3, *B. circulans* 251 CGTase numbering), resulted in an increase in hydrolytic activity and a decrease in cyclization activity (van der Veen *et al.*, 2001, Leemhuis *et al.*, 2002). In branching enzyme of *E. coli* the residue E459 immediately following the (putative) catalytic E458 is important for substrate specificity and activity (Binderup & Preiss, 1998, Abad *et al.*, 2002).

Our mutant data indicate that the H1065 and A1066 residues in conserved region III are important in controlling the hydrolysis/transferase activity ratio (initial rates) in GTFA but have no influence on glucosidic linkage specificity. Conceivably, these residues (corresponding to G261 and V262, close to subsites +2/+3, in *B. circulans* 251 CGTase) are further away from the -1 and +1 subsites. We conclude that the structural features responsible for the larger amount of α-(1→4) glucosidic linkages in glucan and oligosaccharide products synthesized by GTFBIO, compared to the products of GTFA (Kralj *et al.*, 2004a), are most likely outside the three conserved regions investigated in this study (Fig. 1).

Mutations in conserved region IV

Changing the tripeptide C-terminal of the catalytic D1133 in GTFA to N1134S:N1135E:S1136V resulted in a drastic reduction in affinity for sucrose, type of oligosaccharide synthesized, and distribution of glucosidic linkages in the oligosaccharide and glucan products synthesized (this study). This indicates that conserved region IV is important in sucrose binding/processing, consistent with the suggested role of D1133 as transition state stabilizer (MacGregor *et al.*, 1996, Kralj *et al.*, 2004d) (Fig. 1).

Dextransucrase (DSRS) from *Ln. mesenteroides* NRRL B-512F synthesizes panose from sucrose plus maltose (Monchois *et al.*, 1997). Also other members of the panose

series are synthesized by DSRS, with isomaltodextrin chains of increasing DP linked to the non-reducing end of maltose (Monchois *et al.*, 1997, Robyt & Walseth, 1978). GTFA wild type only synthesized panose (Kralj *et al.*, 2004d, Kralj *et al.*, 2004a), whereas all four mutant enzymes containing the triple amino acid mutation N1134S:N1135E:S1136V synthesized a new DP4 oligosaccharide from sucrose and maltose, identified as α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose; α-(1→6)-panose (Fig. 2). Members of the panose series with an increasing DP could not be detected with the method of analysis used in the present study. The synthesis of α-(1→6)-panose by GTFA mutant N1134S:N1135E:S1136V, however, clearly indicates that, with respect to oligosaccharides synthesized from maltose, the GTFA mutants in conserved region IV have an increased α-(1→6) glucosidic bond specificity.

Dextransucrase from *Ln. mesenteroides* NRRL B-512F incubated with sucrose and isomaltose synthesizes isomaltotriose as main product, followed by isomaltooligosaccharides with an increasing DP (Robyt & Eklund, 1983). The amount of isomaltooligosaccharide product in the series decreased with increasing DP. Whereas GTFA wild type incubated with sucrose and isomaltose synthesizes minor amounts of isomaltotriose and isomaltotetraose, GTFA derivatives containing mutation N1134S:N1135E:S1136V clearly produced isomaltotriose and isomaltotetraose (triple and quadruple mutants) and a series of isomaltooligosaccharides up to DP6 (quintuple and sextuple mutants) (Table 5; Fig. 3). Also the oligosaccharide yields with sucrose and isomaltose as acceptor reaction substrate increased drastically (2 fold) with these four GTFA derivatives (Table 5).

A similar strong shift in glucosidic bond specificity also was observed in the glucan products synthesized by the four GTFA N1134S:N1135E:S1136V mutants, reaching levels of 85% of α-(1→6) linkages and very low amounts of α-(1→4) linkages, in the quintuple and sextuple mutants (Table 6; Fig. 4). The data thus show that, with respect to both oligosaccharide and glucan synthesis, the combination of mutations in conserved regions II and IV of GTFA of *Lb. reuteri* 121 successfully transformed the enzyme from a reuteransucrase into a dextransucrase.

In amylosucrase the Asp394 residue next to the catalytically important His392 and Asp393 residues (Sarcabal *et al.*, 2000) is part of acceptor binding site +1 and involved in the correct positioning of the glucosyl residue at this site (Albenne *et al.*, 2004). Mutagenesis of this residue resulted in changes in the product spectrum (mono and oligosaccharides) of amylosucrase from sucrose (Albenne *et al.*, 2004).

Mutant Met329Thr in acarviosyltransferase (ATase) of *Actinoplanes sp.* strain SE50, immediately next to the catalytic Asp 328 (CGTase *B. circulans* 251 numbering), also resulted in changed reaction specificity (10 × higher transferase activity on maltotetraose) (Leemhuis *et al.*, 2004).

The drastic changes observed in the mutant enzymes with respect to affinity for sucrose, type of oligosaccharide and glucan products synthesized, strongly support the hypothesis that residues in this conserved region IV are involved in acceptor substrate binding in GTF enzymes. The precise roles of residues involved remain to be determined.

Conclusion

Amino acid residues and conserved enzyme regions determining the linkage specificity of glucan and oligosaccharide products formed by GTF enzymes have been identified. By rational design we were able to construct a GTFA triple amino acid mutant (N1134S:N1135E:S1136V) with a significant preference for synthesis of α-(1→6) glucosidic linkages over α-(1→4) glucosidic linkages in its oligosaccharide and glucan products, whereas the wild type favors synthesis of α-(1→4) glucosidic linkages over α-(1→6) glucosidic linkages in its oligosaccharide and glucan products. These data show that GTFA was transformed from a reuteransucrase into a dextransucrases type of enzyme. Furthermore, we show for the first time that two distantly located amino acid residues from a glucansucrase enzyme both affect linkage type distribution, in glucan as well as in oligosaccharide products. Although there are clear differences in glucosidic linkages in the glucan products synthesized by the different (mutant) GTFA enzymes, the precise glucan structures remain to be elucidated.

The combined data indicates that glucan and oligosaccharide acceptor substrates in GTFA are elongated at the same acceptor site. Further investigation of the precise roles of amino acid residues involved in acceptor substrate binding in glucansucrases that synthesize other types of glucosidic linkages may serve to expand the range of glucans and glucooligosaccharides that can conveniently be synthesized. Clear understanding of the structural features in glucansucrase enzymes that determine the nature and ratio of glucosidic linkages synthesized eventually may allow production of tailor-made glucan and oligosaccharide products for diverse applications.

ACKNOWLEDGEMENTS

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Chapter 6

Efficient screening methods for glucosyltransferase genes in *Lactobacillus* strains

S. Kralj, G.H. van Geel-Schutten, M.J.E.C. van der Maarel and L. Dijkhuizen

Biocatalysis and Biotransformation (2003) **21**: 181-187

SUMMARY

Limited information is available about homopolysaccharide synthesis in the genus *Lactobacillus*. Extracellular glucosyltransferase (GTF) enzyme activity, resulting in α-glucan synthesis from sucrose, was detected in various lactobacilli. PCR with degenerate primers based on homologous boxes of known glucosyltransferase (*gtf*) genes of *Leuconostoc* and *Streptococcus* strains allowed cloning of fragments of 10 putative *gtf* genes from 8 different glucan producing *Lactobacillus* strains (5 *Lactobacillus reuteri* strains, 1 *Lactobacillus fermentum* strain, 1 *Lactobacillus sakei* strain, and 1 *Lactobacillus parabuchneri* strain). Sequence analysis revealed that these lactobacilli possess a large variation of (putative) *gtf* genes, similar to what has been observed for *Leuconostoc* and *Streptococcus* strains. Homologs of GTFA of *Lb. reuteri* 121 (synthesizing reuteran, a unique glucan with α-(1→4) and α-(1→6) glucosidic bonds) (Kralj *et al.*, 2002) were found in three of the four other *Lb. reuteri* strains tested. The other *Lactobacillus* GTF fragments showed the highest similarity with GTF enzymes of *Leuconostoc* spp.

INTRODUCTION

Various lactic acid bacteria employ large extracellular enzymes, glucosyltransferases (EC 2.4.1.5, commonly named glucansucrases, GTFs), for the synthesis of high molecular weight α-glucans from sucrose. The GTF enzymes of oral streptococci and the dextran- and alternansucrases from *Leuconostoc mesenteroides* strains have been studied in most detail. All GTFs from lactic acid bacteria share a common structure and are composed of four distinct domains (Fig. 1): their N-terminal end starts with (i) a signal peptide, followed by (ii) a highly variable stretch, (iii) a highly conserved catalytic or sucrose binding domain of about 1,000 amino acids (Fig. 2), and (iv) a C-terminal glucan binding domain, composed of a series of tandem repeats (Monchois *et al.*, 1999d).

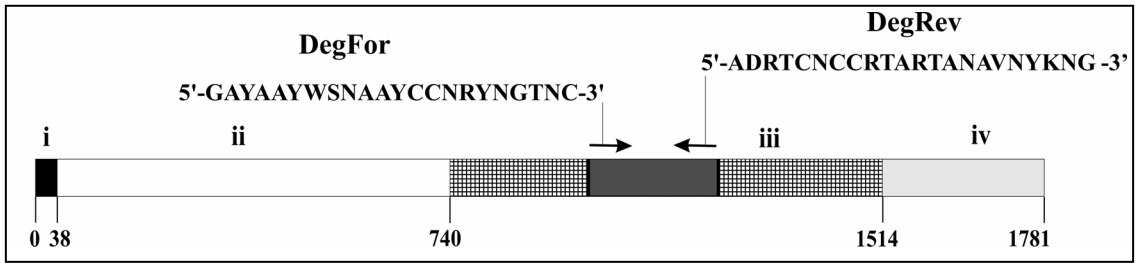

Figure 1. Degenerate primer sequences and their annealing sites in the catalytic domain of GTFA of *Lb. reuteri* 121. The four different domains shown are: i) N-terminal signal sequence; ii) variable region; iii) catalytic domain; iv) C-terminal (putative) glucan binding domain.

There are only few reports about α-glucan synthesis in lactobacilli (Dunican & Seeley, Jr., 1963, Hammond, 1969, Sidebotham, 1974, van Geel-Schutten *et al.*, 1998, van Geel-Schutten *et al.*, 1999). Only the GTFA enzyme responsible for reuteran (a glucan with α-(1→4) and α-(1→6) glucosidic bonds) synthesis in *Lactobacillus reuteri* strain 121 has been subjected to biochemical and molecular characterization (Kralj *et al.*, 2002).

Bacterial Strain	Main α-linkages in glucan polymer			A		B		C
I				⇓ ∇∇		⇓∇∇∇		♦⇓
S. mutans GS5	1→3	GTFB	443	ANFDSIRVDAVDNVDADLLQI	484	HLSILEAWSDNDTPY	555	YSFIRAHDSEVQDLI
S. mutans GS5	1→6	GTFD	457	ANFDGVRVDAVDNVNADLLQI	498	HLSILEAWSDNDPQY	577	YIFIRAHDSEVQTVI
S. downei MFe28	1→3	GTFI	445	ANFDSIRVDAVDNVDADLLQI	486	HVSIVEAWSDNDTPY	557	YSFARAHDSEVQDLI
S. salivarius ATCC 25975	1→3	GTFJ	463	ANFDGIRVDAVDNVDADMLQL	504	HISVLEAWSLNDNHY	605	YVFIRAHDNNVQDII
S. salivarius ATCC 25975	1→6	GTFK	453	AHFDGIRVDAVDNVSVDMLQL	494	NISILEAWSHNDPYY	575	YLFVRAHDSEVQTVI
S. downei MFe28	1→6	GTFS	429	ANFDGVRVDAVDNVNADLLQI	470	HLSILEAWSGNDNDY	540	YVFIRAHDSEVQTRI
Ln. mesenteroides NRRL B-1299	1→6	DSRB	525	ANFDGIRVDAVDNVDADLLQI	566	HLSILEDWSHNDPEY	637	YSFVRAHDSEVQTVI
Ln. mesenteroides NRRL B- 512F	1→6	DSRS	543	ANFDGIRVDAVDNVDADLLQI	584	HLSILEDWSHNDPLY	759	YSFVRAHDSEVQTVI
Ln. mesenteroides NRRL B-1355	1→6 / 1→3	ASR	626	ANFDGIRVDAVDNVDADLLKI	667	HLSILEDWNGKDPQY	758	YSFVRAHDYDAQDPI
Ln. mesenteroides NRRL B-1299	1→6	DSRE CD1	519	ANFDGYRVDAVDNVDADLLQI	560	HISILEDWDNNDSAY	631	YAFIRAHDSEVQTVI
Ln. mesenteroides NRRL B-1299	1→2	DSRE CD2	2202	ANFDSIRIDAVDFIHNDTIQR	2243	HISLVEAGLDAGTST	2315	YSIIHAHDKGVQEKV
II								
Lb. reuteri 121	1→4 / 1→6	GTFA	1016	ANFDSVRVDAPDNIDADLMNI	1056	HINILEDWNHADPEY	1126	YSFVRAHDNNSQDQI
Lb. reuteri 104R	ND	GTF104R		ANFDGIRVDAVDNVDVDLLSI		HINILEDWGWDDPAY		YNFVRAHDSNAQDQI
Lb. reuteri 180	ND	GTF180		ANFDGIRVDAVDNVDVDLLSI		HINILEDWGWDDPAY		YNFVRAHDSNAQDQI
Lb. reuteri ML1	ND	GTFML1		ANFDSIRVDAVDNVDADLLDI		HINILEDWGGQDPYY		YSFIRAHDNGSQDDI
Lb. parabuchneri 33	ND	GTF33		ANFDGYRVDAVDNVDADLLNI		HLSILEDWDNNDPAY		YTFIRAHDSEVQTII
Lb. sakei Kg15	ND	GTFKg15		ANFDSVRVDAVDNVDADLLNI		HLSILEDWGHNDPLY		YSFVRAHDSEVQTVI
Lb. fermentum Kg3	ND	GTFKg3		ANFDAIRIDAVDNVDADLLQL		HLSILEDWSHNDPAY		YSFVRAHDSEVQTVI
Lb. reuteri ML1	ND	GTFML4		GNFDGFRVDAADNIDADVLDQ		HLSYNEGYHSGAAQM		WSFVTNHDQR-KNLI
Lb. reuteri 121	ND	GTFB		DNFDGFRVDAADNIDADVLDQ		HLSYNEGYHSGAAQM		WSFVTNHDQR-KNLI
Lb. reuteri DSM 20016	ND	GTFDSM		GNFDGFRVDAADNIDADVLDQ		HLVYNEGYHSGAARM		WSFVTNHDQR-KNVI
III								
Ln. citreum 86	ND	GTF86-1		ANFDEIRVDAVDNVDADLLQI		HLSILEDWSHNDPEY		YSFVRAHDSEVQTVI
Ln. citreum 86	ND	GTF86-5		ANFDSIRVDAVDNVDADLLDI		HISILEDWSGLDPNE		YSFVRAHDSEVQGII
Ln. citreum 86	ND	GTF86-8		ANFDGYRVDAVDNVDADLLQI		HISILEDWDNNDSAY		YAFIRAHDSEVQTVI
				:** *:** * : * :.		:: *		: : ** : :

Figure 2. Amino acid sequence alignment of highly conserved stretches (A, B, C) in catalytic domains of glucosyltransferases of lactic acid bacteria (also see (Monchois *et al.*, 1999d) (Bozonnet *et al.*, 2002, Monchois *et al.*, 1999d). I: Published sequences of dextran-, mutan- and alternansucrases of *Leuconostoc* and *Streptococcus* strains. GTFB, *S. mutans* GS5 (Shiroza *et al.*, 1987); GTFD, *S. mutans* GS5 (Honda *et al.*, 1990); GTFI, *S. downei* Mfe28 (Ferretti *et al.*, 1987); GTFJ, *S. salivarius* ATCC 25975 (Giffard *et al.*, 1991); GTFK, *S. salivarius* ATCC 25975 (Giffard *et al.*, 1993); GTFS, *S. downei* MFe28 (Gilmore *et al.*, 1990); DSRB, *Ln. mesenteroides* NRRL B-1299 (Monchois *et al.*, 1998a); DSRS, *Ln. mesenteroides* NRRL B-512F (Monchois *et al.*, 1997); ASR, *Ln. mesenteroides* NRRL B-1355 (Arguello-Morales *et al.*, 2000); DSRE CD1 and CD2, *Ln. mesenteroides* NRRL-B1299 (Bozonnet *et al.*, 2002); II: Sequences of *Lactobacillus* glucosyltransferases, previously published or determined in this study. GTFA, *Lb. reuteri* 121 (Kralj *et al.*, 2002); GTFB, *Lb. reuteri* 121; GTF104R, *Lb. reuteri* 104R; GTF180, *Lb. reuteri* 180; GTFML1, *Lb. reuteri* ML1; GTF33, *Lb. parabuchneri* 33; GTFKg15, *Lb. sakei* Kg15; GTFKg3, *Lb. fermentum* Kg3; GTFML4, *Lb. reuteri* ML1; III: Sequences of *Leuconostoc citreum* 86 glucosyltransferases determined in this study: GTF86-1; GTF86-5; GTF86-8. -, sequence gap; ***** , identical residue; **:** , highly conserved residue; **.** , conserved residue; ⇓, putative catalytic residue; ∇, residue possibly playing a role in binding of acceptor molecules and in the transfer of the glucosyl residue; ♦, putative residue stabilizing the transition state; ND, not determined.

This paper describes the cloning, identification and characterization of (parts of) 10 *gtf* genes from various lactobacilli. The data show that a diversity of *gtf* genes is present in the genus *Lactobacillus*, as is the case in other genera of lactic acid bacteria (*Leuconostoc* and *Streptococcus*).

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions

Strains previously identified as homopolysaccharide or heteropolysaccharide producers (van Geel et al. 1998; unpublished information), *Lactobacillus reuteri* 121 (LB 121), *Lactobacillus reuteri* 180 (LB 180), *Lactobacillus reuteri* ML1 (LB ML1), *Lactobacillus reuteri* 104R (LB104R), *Lactobacillus fermentum* Kg3 (LB Kg3), *Lactobacillus sakei* Kg15 (LB Kg15), *Lactobacillus parabuchneri* 33 (LB 33), *Leuconostoc citreum* 86 (LN 86), *Lactobacillus* sp. 181 (LB 181), *Lactobacillus* sp. 182 (LB 182), were obtained from the culture collection of TNO Nutrition and Food Research, Zeist, The Netherlands. The taxonomic position of the various glucan producing strains was identified by 16sRNA analysis (Gendika, The Netherlands). *Lactobacillus plantarum* WCFS1 (LB WCFS1), of which recently the complete genome sequence became available, (Kleerebezem *et al.*, 2003) was obtained from the Wageningen Centre for Food Sciences (WCFS, The Netherlands). The *Lactobacillus reuteri* type strain DSM 20016 (LB DSM) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). All strains were cultivated anaerobically at 37 °C in MRS medium (Difco) (De Man *et al.*, 1960) or in MRS-s medium (MRS-medium with 100 g l^{-1} sucrose instead of 20 g l^{-1} glucose). *Escherichia coli* DH5α (Phabagen) (Hanahan, 1983), and *E. coli* TOP 10 (Invitrogen) were used as hosts for cloning purposes. Plasmid PCR-XL-TOPO (Invitrogen) was used for cloning purposes. *E. coli* strains were grown aerobically at 37 °C in LB medium (Ausubel *et al.*, 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (50 μg ml^{-1} kanamycin). Agar plates were made by adding 1.5% agar to the LB medium.

Isolation of DNA

Total DNA was isolated according to (Nagy *et al.*, 1995), from MRS grown cells. Plasmid DNA of *E. coli* was isolated using a Wizard Plus SV plasmid extraction kit, according to the instructions of the manufacturer (Promega).

Molecular techniques

General procedures for cloning, *E. coli* DNA transformation, DNA manipulations, and agarose gel electrophoresis were as described (Sambrook *et al.*, 1989). Restriction endonuclease digestions were performed as recommended by the enzyme suppliers (New England Biolabs; Roche Biochemicals). Primers were obtained from Eurogentec. Sequencing was performed by GATC (Germany). DNA was amplified by PCR on a DNA Thermal Cycler PTC-200 (MJ Research) using Expand High Fidelity DNA polymerase (Roche Biochemicals). Fragments were isolated from agarose gels using a Qiagen gel extraction kit (Qiagen) following the instructions of the supplier.

Identification and nucleotide sequence analysis of the glucosyltransferase (*gtf*) genes

The *Lactobacillus gtf* genes were isolated by PCR amplification of chromosomal DNA from the different strains, using degenerate primers (DegFor 5'-GAYAAYWSNAAYCCNRYNGTNC-3' and DegRev 5'-ADRTCNCCRTARTANAV NYKNG -3'; Y=T or C, K=G or T, W= A or T, S = C or G, R = A or G, N = inosine), based on conserved amino acid sequences present in the catalytic core (Fig. 1), deduced from the *gtf* genes of *Lb. reuteri* (*gtfA*), *Streptococcus downei* (*gtfS*), *Streptococcus mutans* (*gtfC*), *Streptococcus downei* (*gtfI*), *Streptococcus salivarius* (*gtfK* and *gtfM)*, and *dsrA* of *Ln. mesenteroides* (Kralj *et al.*, 2002, Gilmore *et al.*, 1990, Ueda *et al.*, 1988, Ferretti *et al.*, 1987, Giffard *et al.*, 1993, Simpson *et al.*, 1995b, Monchois *et al.*, 1996).

The PCR conditions for the amplification of the glucosyltransferase genes from the different bacterial strains were as follows: about 100 ng purified DNA as template, 125 pmol of each primer, 2mM dNTP, 10 × reaction buffer, 4.5 mM $MgCl_2$, 0.7 U Expand High Fidelity DNA polymerase (Roche Biochemicals), were used in the final reaction of 25 µl. The PCR reaction started with a denaturation step (95°C, 5 min). The cycle steps for a total of 35 cycles were as follows: denaturation (95°C, 30 sec), annealing (42 °C, 45 sec) and elongation (72 °C, 1 min).

Amplification products of *Lactobacillus* DNA with the expected size of about 660 bp were either i) directly sequenced, or ii) ligated into pCR-XL-TOPO (Invitrogen) and transformed to *E. coli* TOP 10. From ten random clones, plasmid DNA was isolated and analyzed by restriction using *Eco*RI and *Nsi*I. Subsequently five of the ten clones (from each transformation) were sequenced. Determination of the different nucleotide sequences (GATC, Germany) and analysis confirmed *gtf* gene identities.

Dendrogram construction

Amino acid sequences (~ 200 aa of the catalytic core) were aligned with Clustal W 1.74 (Higgins & Sharp, 1988) with a gap opening penalty of 30 and a gap extension penalty of 0.5. Amino acid sequences were obtained from GenBank: DSRB of *Ln. mesenteroides* NRRL B-1299 (AAB95453), DSRS of *Ln. mesenteroides* NRRL B-512F

(AAA53749), DSRE of *Ln. mesenteroides* NRRL B-1299 (AJ430204), GTFA of *Lb. reuteri* strain 121 (AX306822), ASR of *Ln. mesenteroides* NRRL B-1355 (CAB65910), GTFB of *S. mutans* GS5 (AAA88588), GTFS of *S. downei* Mfe28 (AAA26898), GTFK of *S. salivarius* ATCC 25975 (CAA77898), GTFI of *S. downei* Mfe28 (BAA0296), GTFJ of *S. salivarius* ATCC 25975 (CAA77900). The other sequences used were obtained during this study. Tree construction was performed using TreeCon 1.3b (no correction for distance estimation, 100 bootstrap samples, using the neighbor joining algorithm) (van de Peer & De Wachter, 1994).

Activity staining of *Lactobacillus* GTF enzymes

MRS-sucrose media (10 ml) were inoculated with 200 µl overnight culture of *Lactobacillus* strains (LB DSM, LB 121, LB 104R, LB ML1, LB 180, LB 33, LB Kg3, LB Kg15, LB 181, LB 182, LB WCFS1) or *Ln. citreum* 86, and incubated at 37 °C for 8 h. Cells were removed by centrifugation 10,000 × g, and proteins in the supernatants were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (see below). After SDS-PAGE the gel was washed three times (1 min) with demineralized water and incubated overnight at 37 ° in a 50 mM sodium acetate buffer, pH 5.5, containing 1% sucrose, 1% Tween 80 and 1 mM $CaCl_2$. Glucosyltransferase activity was detected by staining the gels for glucans produced with periodate Schiff stain (PAS) as previously described (van Geel-Schutten *et al.*, 1999).

Gel electrophoresis

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) using the Mini-PROTEAN II system (Bio-Rad), with 7.5% polyacrylamide gels. After GTF activity staining, gels were stained for proteins with coomassie BioSafe (Bio-Rad). The High Molecular Weight marker was used as standard (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Screening for GTF positive *Lactobacillus* strains

Supernatants from the twelve different strains: LB DSM, LB 121, LB ML1, LB 104R, LB 180, LB 33, LB Kg3, LB Kg15, LN 86, LB 181, LB 182 and LB WCFS1 were loaded on SDS-PAGE. After incubation in sucrose buffer, GTF activity bands were identified by PAS staining of the glucans produced (van Geel-Schutten *et al.*, 1999). With the exception of LB WCFS1, of which the genome sequence does not contain genes encoding glucansucrases), (Kleerebezem *et al.*, 2003) LB 181 and LB 182 (heteropolysaccharide producers), (van Geel-Schutten *et al.*, 1998) all strains were positive, showing single or more activity bands at approximately 180 KDa (Fig. 3).

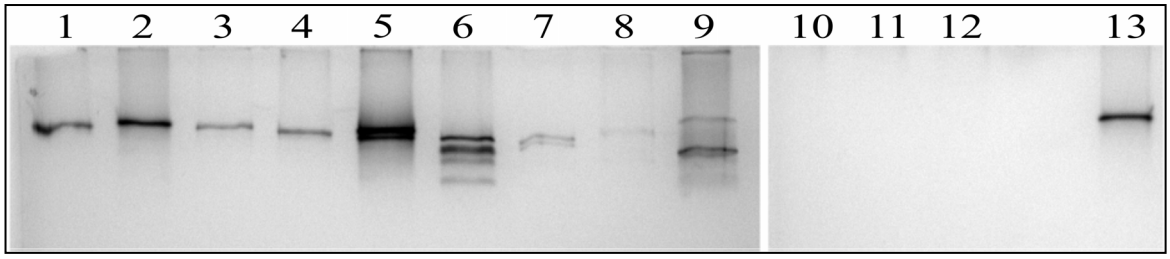

Figure 3. GTFs of different Lactobacilli visualized by SDS-PAGE and PAS staining of the glucans produced. Lanes 1 – 5: *Lb. reuteri* DSM, 121, 104R, ML1 and 180; Lane 6: *Lb. parabuchneri* 33; Lane 7: *Lb. fermentum* Kg3; Lane 8: *Lb. sakei* Kg15; Lane 9: *Ln. citreum* 86; Lane 10 *Lactobacillus* sp. 181; Lane 11 *Lactobacillus* sp. 182; Lane 13 *Lb. plantarum* WCFS1; Lane 14 *Lb. reuteri* 121; Supernatants of the different strains (10 µl) were subjected to SDS-PAGE after overnight incubation at 37°C in MRS-s. (Prior to application, supernatants of *Lb. fermentum* Kg3 and *Lb. reuteri* DSM; *Lb. sakei* Kg15 and *Lb. reuteri* 104R were concentrated 20 × and 50 × times, respectively).

Isolation and nucleotide sequence analysis of parts of the putative *Lactobacillus gtf* genes

Based on sequence homology between conserved regions, located in the catalytic core of different *gtf* genes of lactic acid bacteria, degenerate primers were designed and used for PCR with chromosomal DNA of the nine different strains as template. In each case, except for LB 181, LB 182 and LB WCFS1 fragments of approximately 660 bp were obtained (Fig. 4). PCR products obtained when using chromosomal DNA of LB Kg3 and LB Kg15 as template were sequenced directly, using the same degenerate PCR primers. Sequencing of these two PCR products showed in both cases the presence of only one product. The PCR products from the other seven positive strains (LB DSM, LB 121, LB 104R, LB ML1, LB 180, LB 33, and LN 86) were first ligated in pCR-XL-TOPO (Invitrogen). Subsequently, the six different ligation mixtures were transformed to *E. coli* TOP10. Plasmid DNA was isolated from ten random clones from each transformation. Several *Streptococcal* and *Leuconostoc* species have been shown to contain more than one *gtf* gene (Simpson *et al.*, 1995a, Funane *et al.*, 2000). Therefore, restriction of the different plasmids (60 in total), with *Nsi*I and *Eco*RI, was performed as a first screening to identify differences between the plasmids. Based on the restriction analysis, the inserts of five plasmids were sequenced. Sequence analysis of five plasmids with chromosomal DNA inserts of LN 86 showed the presence of (parts of) three different (putative) *gtf* genes (86-1, 86-5 and 86-8). Strain, LB DSM, LB 104R, LB 180, and LB 33 most likely contain one *gtf* gene each. Strain 121 (*gtfA*, *gtfB*) (Kralj *et al.*, 2002) (this study) as well as strain ML1 contained at least two *gtf* genes (*gtfML1*, *gtfML4*) (Fig. 2B,C). The method used thus allowed detection of several putative *gtf* genes in a single strain.

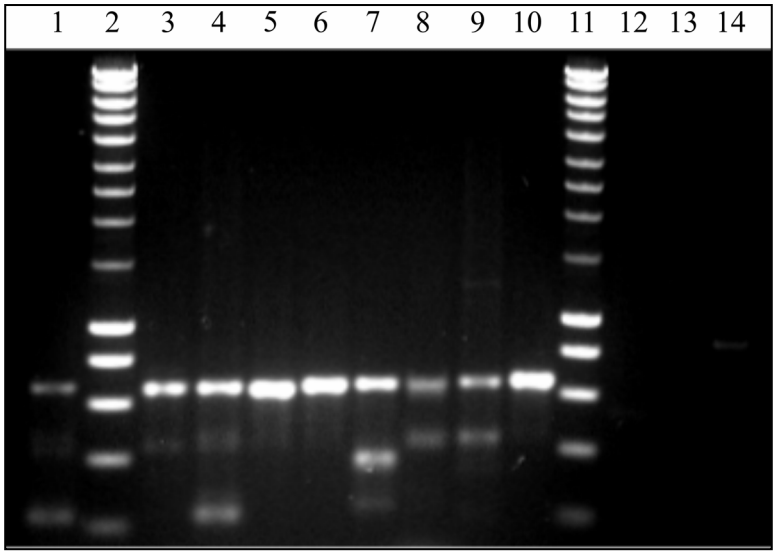

Figure 4. Agarose gel with PCR products obtained with DegFor-DegRev primers and DNAs from PAS-positive and PAS-negative Lactobacilli as templates. Lane 1; and Lanes 3 to 10, glucan-positive Lactobacilli: *Lb. reuteri* DSM 20016, 121, 104R, ML1 and 180; *Lb. parabuchneri* 33; *Lb. fermentum* Kg3, *Lb. sakei* Kg15; Lane 10, *Ln. citreum* 86; Lane 12, 13 and 14 PCR with the glucan-negative strains, *Lactobacillus* sp. 181, *Lactobacillus* sp. 182 and *Lb. plantarum* WCFS1 (van Geel-Schutten *et al.*, 1998, Kleerebezem *et al.*, 2003). Lanes 2 and 11 smart ladder (Eurogentec).

GTF (fragment) sequence comparisons

The amplified products from, LB DSM, LB 121, LB 104R, LB ML1, LB 180, LB 33, LB Kg3, LB Kg15, and LN 86, were all identified as partial sequences of (putative) gtf genes (Fig. 2B,C). The fragments isolated from LB 104R, ML1 (GTFML1) and LB 180 showed highest homology at the amino acid level with GTFA from *Lb. reuteri* 121 (Table 1). The deduced amino acid sequences of the *gtf180* and *gtf104R* encoded (putative) proteins showed very high homology to each other (99% similarity and 99% identity within 206 amino acids). A second (putative) GTF fragment was found in the *Lb. reuteri* strains ML1 (GTFML4) and 121 (GTFB). These fragments showed high homology to each other and to the fragment isolated from *Lb. reuteri* DSM 20016 (GTFDSM) (~80% identity and ~90% similarity). GTF33 showed the highest homology with the first catalytic domain (CD1) encoded by dsrE of *Ln. mesenteroides* NRRL-B1299. GTFKg3 showed the highest homology with DSRB from *Ln. mesenteroides* NRRL-B1299. GTFKg15 showed the highest homology with DSRS of *Ln. mesenteroides* NRRL-B512F (Table 1).

Table 1. Similarities and identities of sequences of the newly isolated GTF fragments to known GTF sequences shown in fig. 5.

Bacterial Strain	(Putative) Protein	Homology to	Genbank	Similarity (%)	Identity (%)
Lb. reuteri DSM 20016	GTFDSM	CD2 DSRE of *Ln. mesenteroides* NRRL B-1299	AJ430204	49	29
Lb. reuteri 104R	GTF104R	GTFA of *Lb. reuteri* 121	AX306822	83	63
Lb. reuteri ML1	GTFML1	GTFA of *Lb. reuteri* 121	AX306822	71	58
Lb. reuteri 180	GTF180	GTFA of *Lb. reuteri* 121	AX306822	83	63
Lb. parabuchneri 33	GTF33	CD1 DSRE of *Ln. mesenteroides* NRRL B-1299	AJ430204	88	79
Lb. fermentum Kg3	GTFKg3	DSRB of *Ln. mesenteroides* NRRL B-1299	AF030129	81	70
Lb. sakei Kg15	GTFKg15	DSRS of *Ln. mesenteroides* NRRL B-512F	U81374	82	72
Lb. reuteri ML1	GTFML4	CD2 DSRE of *Ln. mesenteroides* NRRL B-1299	AJ430204	49	33
Lb. reuteri 121	GTFB	CD2 DSRE of *Ln. mesenteroides* NRRL B-1299	AJ430204	49	33
Ln. citreum 86	GTF86-1	DSRB of *Ln. mesenteroides* NRRL B-1299	AF030129	98	98
Ln. citreum 86	GTF86-5	ASR of *Ln. mesenteroides* NRRL B-1355	Q9RE05	62	49
Ln. citreum 86	GTF86-8	CD1 DSRE of *Ln. mesenteroides* NRRL B-1299	AJ430204	99	99

Dendrogram

Construction of a dendrogram (Fig. 5), based on the partial amino acid sequences (approx. 200 amino acids of the catalytic domains) of GTF enzymes of different lactic acid bacteria, revealed that the fragments of the following putative GTF enzymes (GTF180, GTF104R and GTFML1) isolated from different *Lb. reuteri* strains cluster closely together with GTFA of *Lb. reuteri* 121. Three other putative GTF fragments from *Lb. reuteri* strains (GTFDSM, GTFML4 and GTFB) formed a separate group. The GTF enzymes isolated from the various other lactobacilli cluster with *Leuconostoc* GTF enzymes.

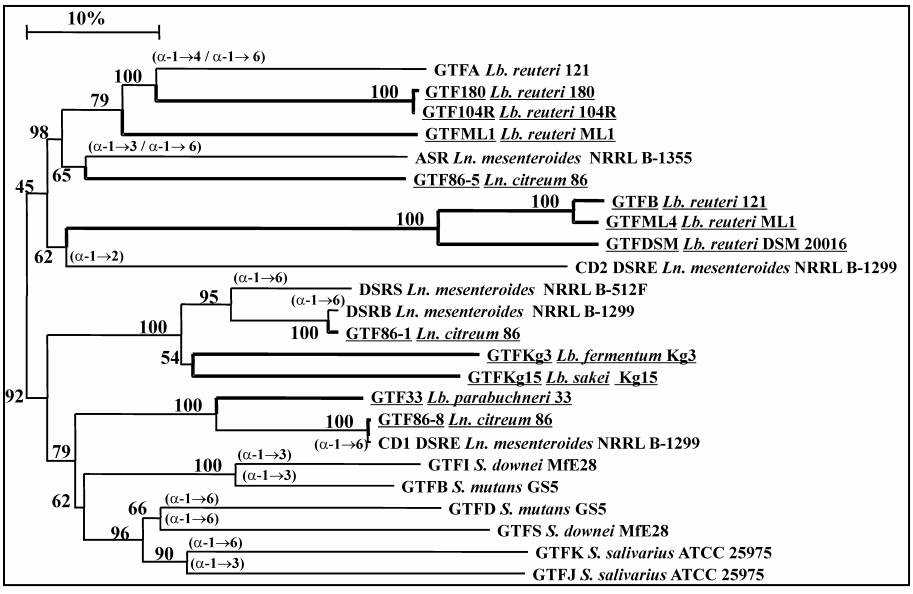

Figure 5. Dendrogram of glucosyltransferases of lactic acid bacteria. The horizontal distances are a measure for the differences at the amino acid level. The length of the upper bar indicates 10% difference. Bootstrap values are given at the root of each branch (in percentages). Sequences of glucosyltransferases determined in this study are indicated with a bold line and are underlined.

Conclusion

SDS-PAGE activity staining for α-glucan synthesis from sucrose, and PCR based cloning of *gtf* gene fragments (catalytic domains), allowed a fairly rapid identification of putative *gtf* genes in several *Lactobacillus* strains. Sequence analysis of the different *gtf* fragments confirmed their identity. Homologs of GTFA of *Lb. reuteri* 121 (Kralj *et al.*, 2002) were detected in three *Lb. reuteri* strains tested. Three other putative GTF fragments from *Lb. reuteri* strains (GTFDSM, GTFML4 and GTFB) formed a separate group. The other partial GTF sequences showed the highest homology to glucosyltransferases from *Leuconostoc* sp. These results show that the large variation of glucosyltransferases in *Leuconostoc* and streptococci sp. can also be found within the lactobacilli. Conceivably, also such a range of different glucosidic bonds may be present in the glucans synthesized by the various GTFenzymes from lactobacilli. Currently, we are cloning and characterizing the full-length *gtf* gene sequences of the various lactobacilli. Many questions still remain to be answered, e.g. about expression, activity, and glucan synthesis of these (putative) GTF enzymes in their individual hosts, and about the number of glucans (and the type of glucosidic bonds present) in the various strains.

ACKNOWLEDGEMENTS

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Chapter 7

Glucan synthesis in the genus *Lactobacillus*: Isolation and characterization of glucansucrase genes, enzymes and glucan products from six different strains

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SUMMARY

Members of the genera *Streptococcus* and *Leuconostoc* synthesize various α-glucans (dextran, alternan, mutan). In *Lactobacillus*, at present only *gtfA* of *Lactobacillus reuteri* 121 has been characterized, the first glucosyltransferase (GTF) enzyme synthesizing a glucan (reuteran) containing mainly α-(1→4) linkages together with α-(1→6) and α-(1→4,6) linkages. Recently, we have detected partial sequences of glucansucrase genes in other members of the genus *Lactobacillus.* Here we report the first time isolation and characterization of dextransucrase and mutansucrase genes and enzymes of various *Lactobacillus* spp. and the glucan products synthesized, with mainly α-(1→6) and α-(1→3) glucosidic linkages.

The four GTF enzymes characterized from three different *Lb. reuteri* strains are highly similar at the amino acid level, and consequently their protein structures are very alike. Interestingly, these four *Lb. reuteri* GTFs have relatively large N-terminal variable regions, containing RDV repeats, and relatively short putative glucan binding domains with conserved and less conserved YG-repeating units. The three other GTF enzymes isolated from *Lactobacillus sakei*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri* contain smaller variable regions and larger putative glucan binding domains when compared to the *Lb. reuteri* GTF enzymes.

INTRODUCTION

Many lactic acid bacteria employ large extracellular glucosyltransferase enzymes (EC 2.4.1.5, commonly named glucansucrases, GTFs), for the synthesis of high molecular mass α-glucans from sucrose. Whereas high similarity exists between these glucansucrase enzymes, they are able to synthesize α-glucans with different types of glucosidic linkages. These glucans can be divided into the following five groups: (i) reuteran, containing large amounts of α-(1→4) glucosidic bonds (*Lactobacillus reuteri* 121) (Kralj *et al.*, 2002), (ii) dextran, containing predominantly α–(1→6) linked glucopyranosyl units in the main chain (Cerning, 1990), (iii) mutan, a polyglucose with mainly α-(1→3) linkages (various streptococci) (Hamada & Slade, 1980), (iv) alternan with alternating α-(1→6) and α-(1→3) linked D-glucopyranosyl units (*Leuconostoc mesenteroides*) (Arguello-Morales *et al.*, 2000), and (v) glucan polymers containing large amounts of α-(1→2) linkages (mainly α-(1→2,6) branching points), produced by *Ln. mesenteroides* strains NRRL-B1299 and a mutant strain (R510) of NRRL B-1355 (Bozonnet *et al.*, 2002, Smith *et al.*, 1998). Within these five distinct groups the glucans may further differ in the nature and amount of other glucosidic linkages present, e.g. of the α-(1→2), α-(1→3), α-(1→4) or

α-(1→6) types, the degree of branching, the type of branching point, e.g. with α-(1→2,6) to α-(1→3,6), or α-(1→4,6) glucosidic linkages, molecular mass, the length of the branching chains and their spatial arrangement (Monchois *et al.*, 1999d).

The distribution of glucosidic linkages has been elucidated for the glucans synthesized by heterologously produced GTF enzymes including (i) 13 GTFs from seven *Streptococcus* strains (Monchois *et al.*, 1999d, Hanada *et al.*, 2002, Konishi *et al.*, 1999), (ii) 7 GTFs from four *Leuconostoc* strains (Monchois *et al.*, 1999d, Bozonnet *et al.*, 2002, Neubauer *et al.*, 2003, Funane *et al.*, 2001, Arguello-Morales *et al.*, 2000), and (iii) *Lb. reuteri* strain 121 (Kralj *et al.*, 2002). Only *gtf* genes encoding either dextran- or mutansucrase enzymes have been characterized in the genus *Streptococcus* (Monchois *et al.*, 1999d, Hanada *et al.*, 2002, Konishi *et al.*, 1999). *Leuconostoc* strains carry *gtf* genes encoding mainly dextransucrase enzymes, but also one alternansucrase encoding gene, and one gene encoding a glucansucrase that synthesizes large amounts of α-(1→2) branch linkages have also been characterized (Arguello-Morales *et al.*, 2000, Bozonnet *et al.*, 2002, Monchois *et al.*, 1999d).

The GTF enzymes of *Streptococcus* sp. are generally produced constitutively (Kim & Robyt, 1994). GTF enzymes of *Leuconostoc* are specifically induced by sucrose. This is disadvantageous for several applications, and therefore some constitutive mutants were constructed (Kim & Robyt, 1994, Kitaoka & Robyt, 1998). Reuteransucrase from *Lb. reuteri* 121 is produced constitutively (van Geel-Schutten *et al.*, 1999). The GTF enzymes from *Lb. reuteri* 180 and *Lb. parabuchneri* 33 are also produced constitutively (unpublished data).

The only streptococcal species that is associated with food technology is *Streptococcus thermophilus*, which is used in the manufacture of yoghurt. The genus *Streptococcus* contains several well-known pathogens (e.g. *Streptococcus pneumoniae*) (Axelsson, 1998, Leroy & de Vuyst, 2004). Furthermore, glucans produced by oral streptococci play a key role in the cariogenesis process, by enhancing the attachment and colonization of cariogenic bacteria (Loesche, 1986). *Leuconostoc* strains play an important role in vegetable fermentations (Axelsson, 1998, Leroy & de Vuyst, 2004).

Lactobacilli are widespread in nature and many species have found applications in the food industry (e.g. dairy products, sourdough) (Axelsson, 1998, de Vuyst & Degeest, 1999). Several *Lb. reuteri* strains are able to produce anti-microbial metabolites (e.g. reutericyclin, reuterin and reutericin), which delay growth of some food borne pathogens (Kabuki *et al.*, 1997, Ganzle *et al.*, 2000, Talarico *et al.*, 1988). Furthermore, some *Lb. reuteri* strains have probiotic properties as has been demonstrated in humans and various animals (Casas *et al.*, 1998, Valeur *et al.*, 2004). The range of glucans and oligosaccharides produced by GTF enzymes present in lactobacilli (Kralj *et al.*, 2004d) may potentially act as prebiotic by stimulating the growth of probiotic strains or of

beneficial endogenic strains of the gastrointestinal tract (Monsan & Paul, 1995, Olano-Martin *et al.*, 2000, Chung & Day, 2002).

Lactobacillus reuteri strains producing glucans thus possess the following general advantages: (i) constitutive GTF enzyme production, (ii) safe (GRAS status), and (iii) potential pro- and prebiotic properties. Glucans and glucooligosaccharides from lactobacilli are therefore interesting and feasible alternatives to the additives currently used in the production of foods (e.g. sourdough, yoghurts, health foods). Although different *Lactobacillus* strains are able to produce glucans (Kralj *et al.*, 2003, Tieking *et al.*, 2003, Sidebotham, 1974, van Geel-Schutten *et al.*, 1998), only the *Lb. reuteri* GTFA enzyme has been characterized thus far. This enzyme synthesizes a highly branched glucan (reuteran) containing α-(1→4) and α-(1→6) linkages (Kralj *et al.*, 2002). The same types of glucosidic linkages were synthesized in its oligosaccharide products (Kralj *et al.*, 2004d). Recently we have shown that lactobacilli in fact contain DNA sequences of other putative glucansucrase genes (Kralj *et al.*, 2003).

This paper describes the characterization of the glucans produced by six different *Lactobacillus* strains, and the isolation and characterization of the corresponding *gtf* genes and GTF enzymes. The data show that members of the genus *Lactobacillus* contain the same variety of *gtf* genes, GTF enzymes and glucan products as *Leuconostoc* and *Streptococcus* spp.

MATERIAL AND METHODS

Bacterial strains, plasmids, media and growth conditions

Lb. reuteri strains 121 (LB 121; LMG 18388), ML1 (LB ML1; LMG 20347) and 180 (LB 180; LMG 18389), *Lactobacillus sakei* Kg15 (LB Kg15), *Lactobacillus fermentum* Kg3 (LB Kg3), and *Lactobacillus parabuchneri* 33 (LB 33; LMG 20349) were obtained from the culture collection of TNO Nutrition and Food Research, Zeist, The Netherlands. All strains were cultivated as described previously (Kralj *et al.*, 2003). *Escherichia coli* TOP 10 (Invitrogen) was used as host for cloning purposes. Plasmids pET15b (Novagen) and pET-101-D-TOPO (Invitrogen) were used for expression of the different *gtf* genes in *E. coli* BL21 Star (DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium (Ausubel *et al.*, 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (100 μg ml^{-1} ampicillin or 50 μg ml^{-1} kanamycin). Agar plates were made by adding 1.5% agar to the LB medium.

Isolation of DNA

Lactobacillus total DNA was isolated according to (Nagy *et al.*, 1995). Plasmid DNA of *E. coli* was isolated using the alkaline lysis method (Birnboim & Doly, 1979) or with a Wizard Plus SV plasmid extraction kit (Promega).

Molecular techniques

General procedures for gene cloning, *E. coli* DNA transformations, DNA manipulations, and agarose gel electrophoresis were as described (Sambrook *et al.*, 1989). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the enzyme suppliers (New England Biolabs; Roche Biochemicals). Primers were obtained from Eurogentec, Seraing, Belgium. Sequencing was performed by GATC (Germany). DNA was amplified by PCR on a DNA Thermal Cycler PTC-200 (MJ Research) using *Pwo* DNA polymerase (Roche Biochemicals) or Expand High Fidelity polymerase (Roche Biochemicals). For inverse PCR (iPCR) the Expand High Fidelity PCR system (Roche Biochemicals) was used as described by the supplier. Fragments were isolated from agarose gels using a Qiagen gel extraction kit (Qiagen) following the instructions of the supplier.

Identification and nucleotide sequence analysis of the glucansucrase genes

A first fragment of the glucansucrase genes was isolated by PCR amplification of chromosomal DNA of the different *Lactobacillus* strains using degenerate primers (DegFor and DegRev) based on sequence similarity between conserved regions, located in the catalytic core of different GTF enzymes of Gram-positive bacteria (see (Kralj *et al.*, 2003). The ~660 bp amplified fragments were used to identify appropriate restriction sites and to design primers for subsequent iPCR reactions (Triglia *et al.*, 1988) (Table 1).

Construction of plasmids

Appropriate primer pairs and template DNA were used to create eight different expression constructs for complete and/or N-terminally truncated and (C-terminally) His-tagged GTF enzymes (Table 2).

Expression and purification of GTF proteins

Cells of *E. coli* BL21star (DE3) harbouring different pET15b or pET-101-D-TOPO derivatives (Table 2) were harvested by centrifugation (10 min at 4 °C at 10,000 × *g*) after 16 h of growth. The pellet was washed with 50 mM phosphate buffer pH 8.0 and the suspension was centrifuged again (10 min at 4 °C at 10,000 × *g*). Pelleted cells were resuspended in 50 mM sodium phosphate buffer pH 8.0 containing 250 mM NaCl, 5 mM β-mercaptoethanol and 10 mM imidazole. Cells were broken by sonication (7 × 15 sec at

Table 1. Primers used for inverse PCR (iPCR) reactions to obtain the 5' or 3' nucleotide sequences of the different *gtf* genes and surrounding regions. Indicated are restriction enzymes used to digest and ligate the chromosomal DNA of the different *Lactobacillus* strains, yielding circular DNA molecules, which were subsequently used as template in iPCR reactions. *[1] For *gtfB* already ~2.4 kb of 3' nucleotide sequence information was available (Kralj *et al.*, 2002). *[2] Another iPCR product obtained, using *Pst*I digested and ligated chromosomal DNA as template, completed the 3'-sequence. *[3] Another iPCR product obtained, using *Nsi*I digested and ligated chromosomal DNA as template, yielded additional 5'-sequence, which was used to design the primers ML4CF and ML4CR. *[4] a-specific iPCR product obtained, providing new sequence information used to design the primers Kg3NF and Kg3N.

Gene	Primer	Sequence Direct primer (5'→3')	Primer	Sequence Reverse primer (5'→3')	Restriction enzyme used	Fragment obtained (bp)
gtfB						
5′	GTFBF	GTTCCGCAAGTTTACTATGGTGACCTTTATAATG	GTFBR	GTTACAAATGACCAGTTTGGCGTAGCTTCATTC	*Sac*I*[1]	5.0
gtfML1						
5′	ML1F	ATTAGATATAGCTGGTGATTACTTTAATGCAG	ML1R	CACGAATGCTATCAAAATTAGCATCAGGATC	*Cla*I	3.5
3′	ML1F2	TTGAAGCATACATTGCAGATCAAAGTAATG	ML1R2	CCCTTTTGCTCTTGCTCAAAGTTAAACTTCG	*Bal*I*[2]	2.0
gtfML4						
5′	ML4F	GTAATTTTGATGGCTTCCGAGTTGATGCTGCTG	ML4R	CTGGATTATACCCCATTAACTTACCATAATTTAG	*Ssp*I*[3]	0.5
3′	ML4F2	GTATGCAATTCTTTTGAGCAATAAAGATACGG	ML4R2	GCCGGAACATTATATTGATCATATTGTTTATTAG	*Ssp*I	2.5
5′	ML4NF	GGCACCGATCAGCTCATGAGAAACTTGGTTGC	ML4NR	CCGACCAGTAATCAACGCGGTTCCCTTCACC	*Ssp*I	1.0
3′	ML4CF	GGTATTGGTTTAGCTGTAAAATTAGCTAATGG	ML4CR	GCAATGATGTCCCATTTTCGTATTTAGCAGACC	*Eco*RI	1.2
gtf180						
5′	180F1	CATGGAGCAAAGTGATGCCAGTGCTAATAAGC	180R1	GTCAACATCTACATTATCAACAGCATCCACTCG	*Kpn*I	1.5
3′	180F2	GCTTATACTATTCTTCTAACAAATAAAGATTCAG	180R2	GGCATATTGTAAAGATTCCACTTCTTATTAG	*Ssp*I	4.5
5′	1802F1	GAAGCAATATGGTAATTTATTGCCGGCTAG	1802R1	GCTTGTGTAACAGTATTTGGCCACCATACC	*Eco*RI	4.0
gtfKg15						
5′	Kg15F1	GGCCACAATGACCCATTATATGTCAAGGACC	Kg15R1	GCGGCTGTAATGTTTAGCAGGTCGGCATCC	*Nsi*I	2.5
3′	Kg15F2	GCTTTGTCCGTGCACACGATAGTGAAGTTC	Kg15F2	GGAATTGCTGGATCAGACGTATTGTCCTTAGC	*Bcl*I	2.0
3′	Kg15CF	AATGGCGATGGTATCGCGTTGAAGGGTTGG	Kg15CR	GTAGAACCAATCCCCGTTTCGGTTTACTGC	*Ase*I	1.5
gtfKg3						
5′	Kg3F1	CGGGATGCCTATGGCATGGCTACAACTGAC	Kg3R1	GAAGTAAATCAGCATCGACATTATCGACCGC	*Ase*I*[4]	0.9
3′	Kg3F2	GGCGCAACCTAGTTACTCGTTTGTGCGTGC	Kg3R2	CATCATTAGCACGGTTGGTGAGATAGAAGTCC	*Bcl*I	2.0
5′	Kg3CF	GGCCAACCGGTGATGACCGGATTCTATCAC	Kg3CR	GCATTTGTACCATTATCACGCAGCACATAGCC	*Sph*I	2.5
3′	Kg3NF	CGACCACTGTTAGCAACTTGGTGGCCAAC	Kg3NR	GGTTGCGATTCGCGCCACGTCTCACCATTTTCC	*Ase*I	1.8
gtf33						
5′	33F1	CGAGTGGACGCTGTGGACAATGTCGATGC	33R1	GCGGTGGGATCATTGGCAGTAATGCTACC	*Bgl*II	6.0
3′	33F2	CCCTAACTCTGACGGATTAACAGTTACTCCCG	33R2	GCGATGATTGTTTGCACTTCACTATCGTGAGCC	*Hpa*I	6.0

Table 2. Primers and expression vectors used for amplification, cloning and expression of the different full length and N-terminally truncated GTF enzymes in *E. coli* BL21 star (DE3). *Sac*I and *Nco*I restriction sites are shown underlined and in italics, respectively. *Apa*I and *Bam*HI restriction sites are shown in bold face and underlined/italics, respectively. *Bgl*II restriction sites are shown in bold face/italics. Sequence coding for His-tag is shown in bold face/italics/underlined. Stop codons are shown in small font. For cloning in pET15b (Novagen), *Nco*I and *Bam*HI/*Bgl*II restriction sites were used. For directional cloning the pET-101-D-TOPO expression vector (Invitrogen) was used.

Construct	Primer	Sequence direct primer (5'→3')	Primer	Sequence reverse primer (5'→3')	Expression vector
GTFB-CHis	GTFBexpF	GGATGCA*CCATGG*ATACAAATACTG GTGATCAGCAAACTGAAC	GTFBexpR	CCTCCTTTCT***AGATct***atta***GTGATG GTGATGGTGATG***GTTGTTAAAGTTTA ATGAAATTGCAGTTGG	pET15b
GTFML1-ΔN-CHis	ML1expCF	GATGCATGAGCTC*CCATGG*GCATTA ATGGTCATCAATATTATATTGACCC	ML1expCR	ATATCGAT**GGGCCC**C***AGATct***atta***G TGATGGTGATGGTGATG***CTTATTAGT ACCACTTAAATCCTGTTGAGTAATTG	pET15b
GTF180-CHis	180expF	GATGCATGAGCTC*CCATGG*ATCAAC AAGTTCAGTCTTCCACAACCC	180expR	ATATCGAT**GGGCCC**C*GGATCc*tatta ***GTGATGGTGATGGTGATG***TTTTTGGC CGTTTAAATCACCAGGTTTTAATGG	pET15b
GTF180-ΔN-CHis	180expCF	GATGCATGAGCTC*CCATGG*GCATTA ACGGCCAACAATATTATATTGACCC	180expR	ATATCGAT**GGGCCC**C*GGATCc*tatta ***GTGATGGTGATGGTGATG***TTTTTGGC CGTTTAAATCACCAGGTTTTAATGG	pET15b
GTFKg15-ΔN-CHis	Kg15expCF	CACCATGATTGCTGGTAAGACGTAT TACTTTGACAAAG	Kg15expCR	ATCTTTTTTATGCTTTTTACTTGTAA ACAACAGACC	pET-101-D-TOPO
GTFKg3-ΔN-CHis	Kg3expF1	CACCATGATTGCAAACAAGGTTTAT GATTTTGATGAG	Kg3expR1	AATCGTCACCAACGTACCAGCACCAG TTTTC	pET-101-D-TOPO
GTF33-CHis	33expF	CACCATGGCCGGCAATAATGATCCG CAGCAGACC	33expR	CTTGATATTGGTTTTAATTACTTTAG TTAATGC	pET-101-D-TOPO
GTF33-ΔN-CHis	33expCF	CACCATGATCAATGGACAATCACTA TATTTCAATAAAAC	33expR	CTTGATATTGGTTTTAATTACTTTAG TTAATGC	pET-101-D-TOPO

7 micron with 30 sec intervals) and centrifuged (10 min at 4 °C at 10,000 × *g*). The clear lysate containing GTF activity was loaded on a Ni-NTA column (Qiagen). Binding was achieved using 50 mM sodium phosphate buffer pH 8.0 containing 250 mM NaCl, 5 mM β-mercaptoethanol and 10 mM imidazole, followed by washing using the same buffer. Elution of the His-tagged protein(s) was performed using 50 mM sodium phosphate buffer pH 8.0 containing 250 mM NaCl, 1 mM β-mercaptoethanol and 200 mM imidazole.

Enzyme assays

Using His-tag purified GTF enzymes from *E. coli* extracts, or culture supernatants of *Lactobacillus* strains grown on MRSs as source of enzyme, GTF total activity was measured by determining the release of fructose from sucrose at 37 °C in 50 mM sodium acetate buffer pH 5.5 containing 1 mM $CaCl_2$ and 100 mM sucrose (van Geel-Schutten *et al.*, 1999).

SDS-PAGE followed by activity staining

Gel electrophoresis and GTF activity staining of gels with periodic acid Schiff reagens was performed as described previously (Kralj *et al.*, 2003).

Production of glucans and analysis

(i) Polymer production. Glucans synthesized by cultures of the *Lactobacillus* strains, and glucans synthesized by the His–tag purified GTF enzymes from *E. coli* were produced and isolated by ethanol precipitation as described previously (Kralj *et al.*, 2002).

(ii) Methylation. Polysaccharides were permethylated using methyl iodide and dimsyl sodium ($CH_3SOCH_2^-$-Na^+) in DMSO at room temperature (Hakomori, 1964). After hydrolysis with 2 M trifluoroacetic acid (1 h, 125 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ (Harris *et al.*, 1984). Mixtures of partially methylated alditol acetates obtained were analyzed by GLC on a CP Sil 5 CB column (25 m × 0.53 mm; Chrompack) and by GLC-mass spectrometry (MS) on a RTX 5 Sil MS (30 m × 0.25 mm; Restek) column (Chaplin, 1982, Jansson *et al.*, 1976).

(iii) Molecular masses of the glucans. Molecular mass analysis was performed as described previously, using high performance size exclusion chromatography (HPSEC) coupled on-line with a multi angle laser light scattering (MALLS) and differential refractive index detection (Kralj *et al.*, 2002).

Nucleotide accession numbers

The nucleotide sequences of the six different *Lactobacillus gtf* genes and their flanking regions have been assigned the following GenBank accession no's: *gtf180*, AY697430; *gtfML1*, AY697431; *gtf33*, AY697432; *gtfKg3,* AY697433; *gtfKg15*, AY697434; *gtfB*, AY697435.

RESULTS AND DISCUSSION

Isolation and nucleotide sequence analysis of six putative *Lactobacillus* glucansucrase genes

Previous work showed that a second putative *gtf* gene was located upstream of *gtfA* of *Lb. reuteri* 121 (Kralj *et al.*, 2002). Part of this putative *gtfB* gene was isolated from LB 121 chromosomal DNA using degenerate primers (Kralj *et al.*, 2003). Using iPCR, the complete *gtfB* sequence was obtained in the present study (Table 1, Fig. 1).

From five other *Lactobacillus* strains, parts of six putative *gtf* genes (*gtfML1*, *gtf180*, *gtfKg15*, *gtfKg3*, *gtf33*, and *gtfML4*) could be isolated (Kralj *et al.*, 2003, van Geel-

Schutten, 2003). In subsequent steps the complete nucleotide sequences of the different *gtf* genes (except *gtfML4*) was obtained using the iPCR method (Fig. 1, Table 1).

The *gtf* genes in the different *Lactobacillus* species/strains appear to have different chromosomal locations, with a relatively high frequency of transposase homologs flanking the different *gtf* genes (Fig. 1, Table 3).

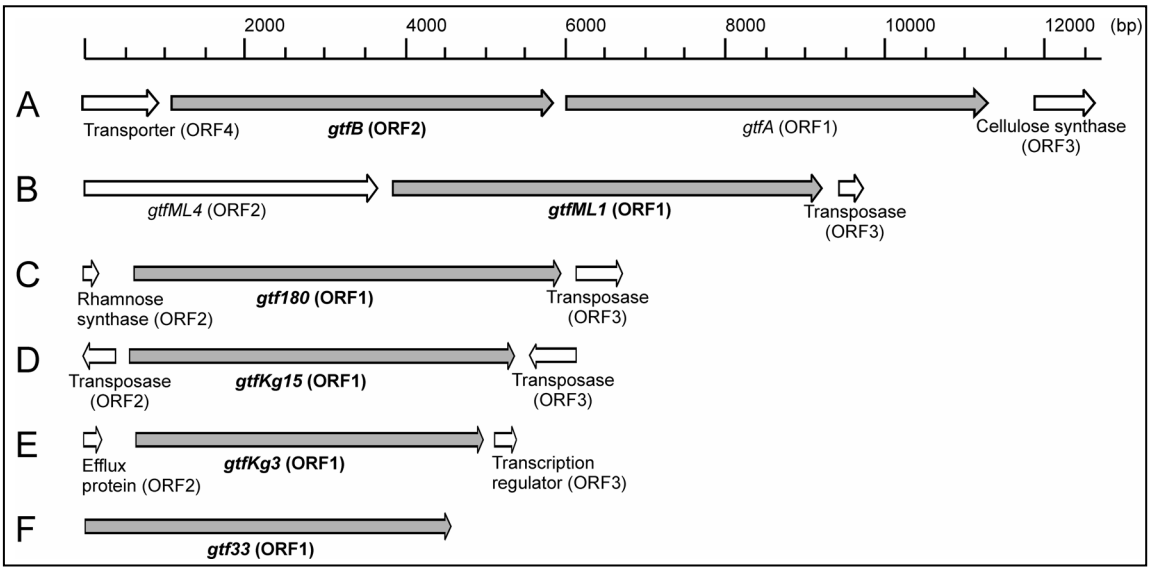


Figure 1. Overview of the size and organization of DNA fragments, isolated by inverse PCR, carrying the different *gtf* genes and surrounding regions from six different *Lactobacillus* strains characterized in this study (shown in bold type): A) *gtf*A (characterized previously, Kralj *et al.*, 2002) and *gtfB* from *Lactobacillus reuteri* 121; B) *gtfML1* and (partly) *gtfML4* from *Lactobacillus reuteri* ML1; C) *gtf180* from *Lactobacillus reuteri* 180; D) *gtfKg15* from *Lactobacillus sakei* Kg15; E) *gtfKg3* from *Lactobacillus fermentum* Kg3; F) *gtf33* from *Lactobacillus parabuchneri* 33. Partial ORFs are indicated with open arrows.

Amino acid sequence analysis of the six isolated GTFs

Alignment of the deduced amino acid sequence of the different GTF enzymes with other glucansucrases using Blast (Altschul *et al.*, 1990), revealed clear similarities to other GTF enzymes of lactic acid bacteria (Table 3). The four GTF enzymes characterized of 3 different *Lb. reuteri* strains are highly similar (Kralj *et al.*, 2002)(This study, Table 3 and Fig. 2). *Lb. sakei* GTFKg15 and *Lb. fermentum* GTFKg3 displayed highest identity and similarity with *Ln. mesenteroides* Lcc4 DSRD. *Lb. parabuchneri* GTF33 was found to have highest similarity with CD1 of DSRE of *Ln. mesenteroides* NRRL B-1299. Analysis of the deduced GTF amino acid sequences encoded by the six completely isolated *gtf* genes revealed the presence of: (i) a signal peptide, (ii) a highly variable stretch, (iii) a highly conserved catalytic domain, and (iv) a C-terminal domain often referred to as a glucan binding domain (GBD; Fig. 2) (Monchois *et al.*, 1999d).

Signal peptides. Consistent with the extracellular location of GTF enzymes, all the sequences contain a typical gram-positive signal peptide ranging in size from 37 to 46 amino acids (Fig. 2). The predicted cleavage sites were located using the Signal P server (http://www.cbs.dtu.dk/services/SignalP/).

Table 3. Overview of the highest identity and similarity scores of the different GTF enzymes and surrounding ORFs, isolated from the various *Lactobacillus* strains, to proteins present in databases (see also Fig. 1). *, Partial open reading frames; *f*, No. of amino acids within which the identity and similarity applies.

Lactobacillus strain	(Putative) proteins	Highest similarity to	Genbank no.	Identity (%)	Similarity (%)	No. of amino acids *f*
Lb. reuteri 121	GTFA	ASR from *Ln. mesenteroides* NRRL B-1355	AJ250173	47	60	1261
	GTFB	GTFA from *Lb. reuteri* 121	AX306822	45	60	1677
	ORF3*	cellulose synthase from *Populus tremuloides*	AY095297	28	47	87
	ORF4*	putative transporter from *Streptococcus thermophilus*	AF454500	26	48	333
Lb. reuteri ML1	GTFML1	GTFA from *Lb. reuteri* 121	AX306822	78	86	1775
	GTFML4*	GTFB from *Lb. reuteri* 121	This study	90	92	1231
	ORF3*	transposase from *Lb. reuteri* plasmid	AF449484	98	99	102
Lb. reuteri 180	GTF180	GTFA from *Lb. reuteri* 121	AX306822	78	87	1768
	ORF2*	dTDP L-rhamnose synthase from *Streptococcus pneumoniae*	AF246897	64	74	56
	ORF3*	putative transposase from *Enterococcus faecium*	U63997	37	56	181
Lb. sakei Kg15	GTFKg15	DSRD from *Leuconostoc mesenteroides* Lcc4 GTFA from *Lb. reuteri* 121	AY017384 AX306822	57 45	69 57	1336 1111
	ORF2*	putative transposase from *Streptococcus criceti* E49	BAB64015	36	51	132
	ORF3*	putative transposase from *Leuconostoc lactis*	JC4048	49	69	188
Lb. fermentum Kg3	GTFKg3	DSRD from *Ln. mesenteroides* Lcc4 GTFA from *Lb. reuteri* 121	AY017384 AX306822	51 40	65 54	1475 1303
	ORF2*	putative efflux protein from *Streptococcus pyogenes* SSI-1	AP005145	29	56	57
	ORF3*	alkaline phosphatase synthesis transcriptional regulatory protein from *Staphylococcus aureus* MW2	AP004827	50	73	86
Lb. parabuchneri 33	GTF33	CD1 of DSRE from *Ln. mesenteroides* NRRL B-1299 GTFA from *Lb. reuteri* 121	AJ430204 AX306822	68 49	76 65	1261 1029

N-terminal variable regions. The protein structures of GTFB, GTF180 and GTFML1 found in three different *Lb. reuteri* strains are very similar to that of GTFA of *Lb. reuteri* 121 (Fig. 2). All three GTFs contain a relatively large and highly similar variable region (~700 amino acids), with 5 RDV-repeats, R(P/N)DV-x_{12}-SGF-x_{19-22}-R(Y/F)S (x, non-conserved amino acid residue), as previously observed in GTFA of *Lb. reuteri* 121 (Kralj *et al.*, 2002) (Fig. 2). The variable domains of the other three isolated GTFs were smaller and contained different repeating units from the *Lb. reuteri* GTFs. GTFKg3 and GTFKg15 contain, respectively, 5 and 6 conserved and less-conserved YG-repeats, NDGYYFxxxGxxHºx(G/N)HºHºHº (x, non-conserved amino acid residue; Hº, hydrophobic amino acid residue), in their variable region (Fig. 2) (Giffard & Jacques, 1994). In the variable region of GTF33, 9 short unique repeating units, designated "TTQ", were found. These repeats were 15 amino acids long, TTTQN(A/T)(P/A)NN(S/G)N(D/G)PQS, and showed no significant similarity to any protein motif present in databases (Fig. 2). Different repeating units also could be identified in the N-terminal variable domain of

other glucansucrases: A-repeats in alternansucrase and dextransucrases of *Ln. mesenteroides* sp. (Janecek *et al.*, 2000), motif T, TDD**KA**(A/T)**TT**A(A/D)**T**S (boldface type indicates conserved amino acids) in DSRT of *Ln. mesenteroides* NRRL B-512F (Funane *et al.*, 2000), motif S, PA(A/T)**DKA**VD**TT**P(A/T)**T**, boldface type indicates conserved amino acids) in DSRE of *Ln. mesenteroides* NRRL B-1299 (Bozonnet *et al.*, 2002), and RDV repeats in GTFA of *Lb. reuteri* 121) (Kralj *et al.*, 2002). However, deletion studies of the variable domain showed that it does not determine the type of glucosidic linkages present or glucan size (only determined for GTFA) of the synthesized glucans (Monchois *et al.*, 1999a, Kralj *et al.*, 2004d).

Catalytic domains. The catalytic domains of the putative GTF enzymes range in size from 741 to 774 amino acids (Fig. 2). Within all the catalytic domains the three completely conserved amino acids already identified in other GTF enzymes (Asp1024, Glu1061 and Asp1133; GTFA *Lb. reuteri* 121 numbering) as being essential for enzymatic activity could be identified (Devulapalle *et al.*, 1997, Kralj *et al.*, 2003, Kralj *et al.*, 2004d).

Putative glucan binding domains (GBDs). The C-terminal domain of *Streptococcus* and *Leuconostoc* GTF enzymes consists of a series of different tandem repeats, which have been divided into four classes: A, B, C and D repeats. Within the A-D repeats, a repeating unit designated YG can be distinguished (Giffard & Jacques, 1994). GTFB, GTFML1 and GTF180 possess a relatively short GBD of 134-263 amino acids, comparable with the GBD from GTFA of *Lb. reuteri* 121 (Fig. 2), consisting of several conserved and less well conserved YG-repeats (Kralj *et al.*, 2002). Characterization of sequential C-terminal deletion mutants of GTFA revealed that its C-terminal domain has an important role in glucan binding (Kralj *et al.*, 2004d).

The putative GBDs of the other isolated GTFs are approximately twice as large when compared to the *Lb. reuteri* GBDs. They contained a varying number of conserved and less well conserved YG repeating units (Fig. 2) and no A, B, C or D repeats could be identified. GTF33 contains besides the 17 YG-repeats, two unique repeating units designated “KYQ” (49 amino acids, AVK(T/A)A(K/Q)(A/T)(Q/K)(L/V)(A/N)K(T/A)K AQ(I/V)(A/T)KYQKALKKAKTTKAK(A/T)QARK(S/N)LKKA(E/N(T/S)S(F/L)(S/T)K A) that showed no significant similarity to any protein motif present in databases. GTFKg15 possesses an additional stretch at the end of its putative GBD, which shows similarity to part of a putative extracellular matrix binding protein from *Streptococcus pyogenes* M1 (AE006525; 44% similarity and 56% identity within 75 amino acids) (Fig. 2).

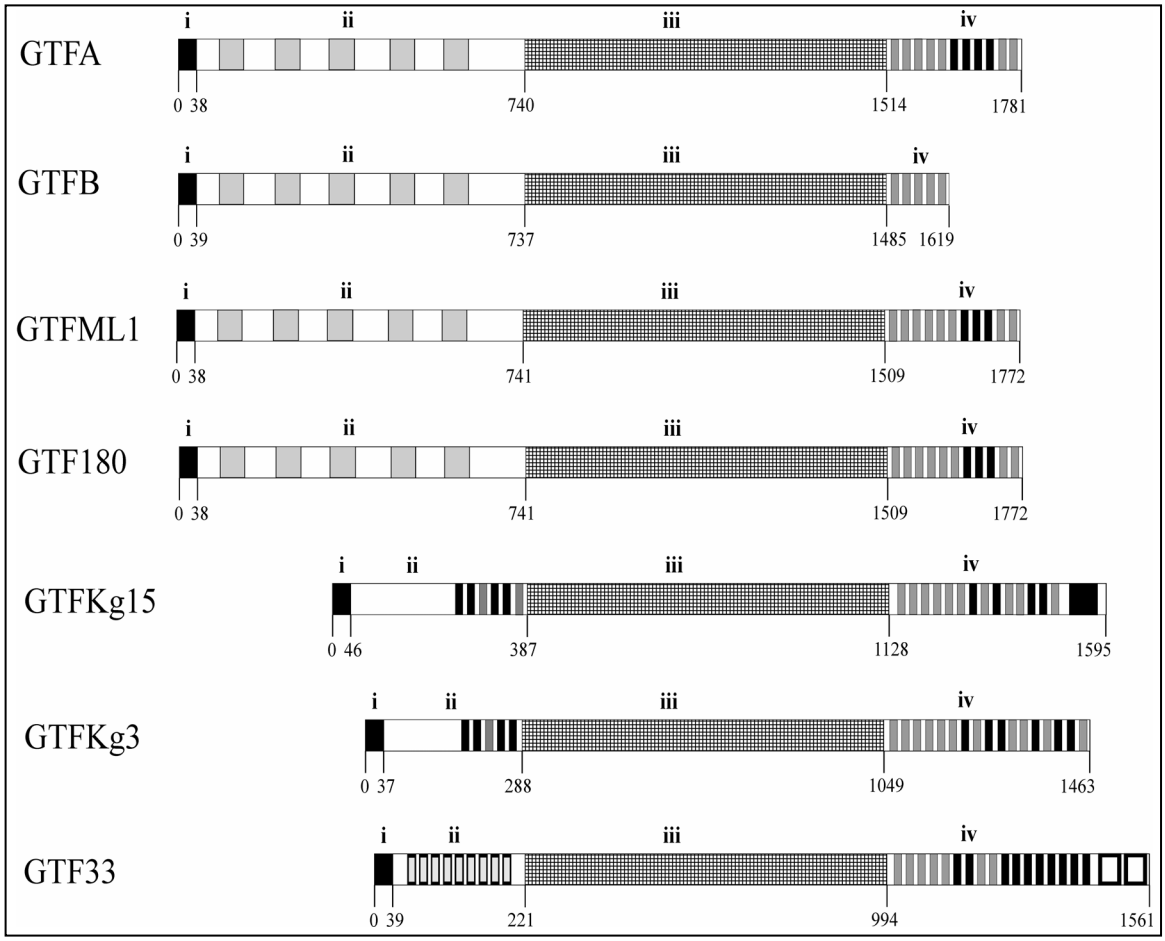


Figure 2. Schematic representation of the organization of GTFA (Kralj *et al.*, 2002) and GTFB of *Lb. reuteri* strain 121, GTFML1 of *Lb. reuteri* ML1, GTF180 of *Lb. reuteri* 180, GTFKg15 of *Lb. sakei* Kg15, GTFKg3 of *Lb. fermentum* Kg3 and GTF33 of *Lb. parabuchneri* 33, showing the four different domains i) N-terminal signal sequence; ii) variable region (RDV repeats are indicated by light grey squared boxes, TTQ repeats in GTF33 are indicated by light grey boxes surrounded by a bold line); iii) catalytic domain; iv) C-terminal (putative) glucan binding domain (GBD; KYQ repeats in GTF33 are indicated with white squared boxes surrounded by a bold line; the domain showing similarity to an extracellular matrix binding protein in GTFKg15 is indicated by a black squared box). Conserved YG-repeats according to the definition of Giffard and Jacques (Giffard & Jacques, 1994), in the N-terminal variable region and/or in the GBD are indicated by black boxes, less conserved YG-repeats are shown as light grey boxes.

Expression of the *gtf* genes in *E. coli*

Based on the nucleotide sequence information obtained, six different *gtf* genes were cloned and expressed in *E. coli* (Table 2). The *gtfB* gene was expressed as a full-length protein. The *gtf180* and *gtf33* genes were expressed as proteins with and without their N-terminal variable regions. The *gtfML1*, *gtfKg3* and *gtfKg15* genes were expressed as proteins without N-terminal variable region (Table 2). Except for GTFB of *Lb. reuteri* 121 His-tag purified GTF proteins showed enzymatic activity, as measured by fructose release from sucrose. SDS-PAGE showed that in all cases protein was present as a band

corresponding to the molecular mass of the different truncated and full length enzymes (data not shown). Staining with Schiff reagents for polymer synthesizing activity was positive with all recombinant enzymes (except for GTFB under the conditions examined). In the supernatant of *Lb. reuteri* 121 grown cultures only the GTFA enzyme was found (under the growth conditions tested). Furthermore, the glucan synthesized by *Lb. reuteri* 121 is identical to the glucan produced by GTFA (Kralj *et al.*, 2002). This suggests that GTFB of *Lb. reuteri* 121 either synthesizes a similar glucan product or (most likely) is not active under the growth conditions tested. GTFB has a relatively small GBD, compared to GTFA (Fig. 2). However, deletion studies with GTFA showed that this enzyme was still active after truncation of the GBD to the size (6 YG repeats deleted) of the GBD of GTFB (Kralj *et al.*, 2004d). GTFB also possesses the three catalytically important residues (Devulapalle *et al.*, 1997, Kralj *et al.*, 2004d). Its inactivity may be caused by the aberrant amino acid sequence at the start of its catalytic core. The highly conserved motif "IN**G**QY**Y**" indicating the start of the catalytic core in GTF enzymes is absent in GTFB. This GTFB region also contains many gaps and overall very poor similarity when compared to other GTF enzymes. Truncations in this region of GTFI of *S. downei* Mfe28 resulted in drastic loss of enzyme activity (Monchois *et al.*, 1999c).

GTFML4 showed high similarity with GTFB of *Lb. reuteri* 121 (Table 3), including the differences with other GTF enzymes listed above. Furthermore, the organization of *gtfML1* and *gtfML4* on the genome of *Lb. reuteri* ML1 was similar to that of *gtfA* and *gtfB* of *Lb. reuteri* 121 (Fig. 1). Consequently, no further efforts were made to clone the full-length *gtfML4* gene. The identical nature of the glucans produced by LB ML1 and by the purified recombinant GTFML1 was confirmed by methylation analysis (Table 4). It appears likely that under the conditions tested also *gtfML4* is not active, as is the case with GTFB.

Analysis of glucans produced by *Lactobacillus* GTFs, N-terminally truncated and full length recombinant GTFs

Supernatants of sucrose grown cultures of the different *Lactobacillus* strains, His-tag purified truncated and full length recombinant GTFs from *E. coli* extracts, were incubated with sucrose and the soluble glucans produced were purified. Methylation analysis showed that the soluble glucans produced by the five *Lactobacillus* strains and the corresponding recombinant GTF enzymes were virtually identical (Table 4). The polymers produced by the different *Lactobacillus* strains are large in size, ranging from $0.2 \cdot 10^6$ to $50 \cdot 10^6$ Da (Table 4). Previous work showed that deletion of the N-terminal variable domain of GTFA from *Lb. reuteri* 121 and GTFI from *S. downei* Mfe28 had no effect on polymer size (only determined for GTFA) and linkage type distribution (Monchois *et al.*, 1999a, Kralj *et al.*, 2004d). To facilitate cloning and reduce enzyme sizes, some of the GTF enzymes were produced without this variable region (ΔN, Table

2). This yielded active GTF enzymes, which synthesized virtually the same glucans as the wild type *Lactobacillus* strains (Table 4). As previously reported (Kralj *et al.*, 2002), GTFA of *Lb. reuteri* 121 synthesized a reuteran [mainly α-(1→4) linkages]. Three *Lactobacillus* strains, LB Kg3, LB Kg15 and LB 33, and their GTF enzymes were characterized as producing dextran [mainly α-(1→6) linkages] like polymers. Strain LB ML1 and GTFML1 produced a highly branched mutan [mainly α-(1→3) linkages] like polymer. Strain LB 180 and GTF180 produced a polymer containing large amounts of α-(1→6) glucosidic linkages, together with lower amounts of α-(1→3) linked glucosyl units (most likely a dextran with large amounts of α-(1→3 linkages).

Table 3. Methylation analysis of the glucans produced from sucrose by GTF enzymes in supernatants of *Lactobacillus* strains (LB) and by His-tag purified complete (rec) or N-terminally truncated (tru) GTF enzymes from *E. coli*. GTFB from *Lb. reuteri* 121 was inactive under the conditions used in this study. ND, Not Determined; *GTFA from *Lb. reuteri* 121 was used as a reference (Kralj *et al.*, 2002, Kralj *et al.*, 2004d).

Linkage type	**121***			**ML1**		**180**			**Kg15**		**Kg3**		**33**		
	LB	**rec**	**tru**	**LB**	**tru**	**LB**	**rec**	**tru**	**LB**	**rec**	**LB**	**rec**	**LB**	**rec**	**tru**
	Methylation (%)														
Terminal	9	11	9	18	17	10	7	8	4	5	3	5	6	3	4
α-(1→3)				47	48	26	32	26	1	1	1	0	9	18	17
α-(1→4)	49	46	46												
α-(1→6)	26	26	34	10	10	51	52	54	86	85	89	87	75	71	70
α-(1→3,6)				26	25	13	10	12	9	9	7	7	9	8	9
α-(1→4,6)	15	17	12												
Molecular mass (1×10^6 Da)															
	40	45	50	8	ND	36	ND	ND	27	ND	24	ND	0.2	ND	ND

Conclusion

This paper reports the first examples, we believe, of isolation and characterization of dextransucrase and mutansucrase genes/enzymes, and dextran/mutan products from *Lactobacillus* spp. The genus *Lactobacillus* thus contains the same variety of *gtf* genes, GTF enzymes and glucan products as found within the genera *Leuconostoc* and *Streptococcus*, plus the ability to synthesize reuteran (reuteransucrase of *Lb. reuteri* 121). GTFA, GTF180 and GTFML1 are highly similar (Table 3) but synthesize glucans with different glucosidic linkages. These enzymes thus are very interesting candidates for structure/function studies aiming to identify amino acid residues responsible for glucosidic bond specificity.

Chapter 8

Summary and concluding remarks

Lactic acid bacteria (LAB) are widely used in the feed and food (fermentation) industry (e.g. beer, cheese, yoghurt, olives, pickles, sauerkraut and silage) (Leroy & de Vuyst, 2004). LAB form various metabolites during fermentation of carbohydrates, which contribute positively to the taste, smell and preservation of the final product. LABs are also known to produce an abundant variety of exopolysaccharides (EPSs). The properties and thus the potential for application of EPSs vary and are dependent on their monosaccharide composition, type of linkages present, degree of branching and molecular weight. Depending on their monosaccharide composition and biosynthetic pathway, EPSs produced by LAB can be divided into two groups, heteropolysaccharides and homopolysaccharides (Jolly *et al.*, 2002, de Vuyst & Degeest, 1999). Heteropolysaccharides are composed of a variety of sugar residues; mainly glucose, galactose, fructose and rhamnose. Intracellular sugar nucleotides, formed from intermediates of central carbon metabolism play an essential role in heteropolysaccharide synthesis. The activated sugar nucleotides are transferred onto a lipophilic carrier, followed by assembly of the oligosaccharide repeating unit by specific glycosyltransferases. Then the repeating unit is exported and polymerized. (Jolly *et al.*, 2002, Welman & Maddox, 2003).

Homopolysaccharides are composed of one type of monosaccharide. We are interested in synthesis of homopolysaccharides from sucrose by sucrase type of enzymes. Products of these enzymes may consist of either glucose (polymer: α-glucan) or fructose (polymer: β-fructan) residues with varying glycosidic linkages, degree of branching and molecular weights. α-Glucans of LAB are synthesized by single (glucan)sucrase enzymes from sucrose (donor). Instead of the growing glucan chain (polymerization), also water (hydrolysis) or other oligosaccharides (acceptor reaction) may be used as acceptor substrates.

The physiological roles of α-glucans in lactic acid bacteria have not been clearly established, and are probably diverse and complex. The polymers may render protection to microbial cells in their natural environment, against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds (Cerning, 1990, Jolly *et al.*, 2002). These polysaccharides may play a role in adhesion of cells to solid surfaces and in biofilm formation (e.g. formation of dental plaque on tooth surfaces by *Streptococcus* sp.) (Russell, 1990). Furthermore, glucans (e.g. dextran) are used in industrial (food) and biomedical applications (e.g, gel filtration products, bakery products, blood plasma substitutes).

The work in this thesis focuses on glucansucrases from lactic acid bacteria, especially lactobacilli. Lactobacilli have been used as probiotics (“mono- or mixed culture of living microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous population of gastrointestinal microorganisms”) (Havenaar & Huis in 't Veld, 1992) to control intestinal disorders such as lactose

intolerance, acute gastroenteritis due to rotavirus and other enteric pathogens, adverse effects of pelvic radiotherapy, constipation, inflammatory bowel disease, and food allergy. The beneficial effects of these organisms have been attributed to their ability to suppress the growth of pathogenic bacteria, possibly by secretion of antibacterial substances such as lactic acid, peroxide and bacteriocins. Their survival during the passage through the human gut, when administered in fermented milk products, has been investigated extensively in recent years.

Prebiotics are defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson & Roberfroid, 1995). Certain oligosaccharides (isomaltooligosaccharides, lactulose, fructans and oligosaccharides containing α-(1→2) glucosidic bonds) and polysaccharides are resistant to digestion by enzymes of the gastro-intestinal tract. These poly- and oligosaccharides can, however, be fermented by beneficial microorganisms**,** such as bifidobacteria. Prebiotics therefore, selectively stimulate the growth of these microorganisms. The *Lactobacillus* strains and their oligosaccharide and glucan products described in this thesis potentially can be used as a synbiotic (combination of pro- and prebiotic).

Glucansucrases or glucosyltransferase (GTF) enzymes of LAB (E.C. 2.4.1.5) are composed of four different domains: their N-terminal end starts with (i) a signal peptide, followed by (ii) a highly variable stretch, (iii) a highly conserved catalytic or sucrose binding domain and (iv) a C-terminal glucan binding domain, composed of a series of tandem repeats (Monchois *et al.*, 1999d).

Glucan polymer synthesis proceeds in a processive manner, intermediate oligosaccharides can not be detected and polysaccharides of high molecular weight are obtained at early reaction times (Tsuchiya *et al.*, 1953, Bovey, 1959, Ebert & Schenk, 1968). The exact mechanisms of glucan synthesis are still not fully understood. Two alternative mechanisms have been proposed for the glucan chain growth: **a**) *Non-reducing end elongation*: this mechanism involves the presence of one site (an aspartate or glutamate) acting as a nucleophilic group and another residue acting as a proton donor, **b**) *Reducing end elongation*: according to this mechanism, the reaction occurs in two steps involving two sucrose binding sites (Monchois *et al.*, 1999d, Robyt, 1996).

A large collection of glucansucrases from *Leuconostoc* and *Streptococcus* sp. has been isolated over the years. Glucansucrases are able to synthesize a diversity of α-glucans with α-(1→6) (dextran by dextransucrases (DSR), mainly found in *Leuconostoc*), α-(1→3) (mutan by mutansucrase, mainly found in *Streptococcus*), alternating α-(1→3) / α-(1→6) (alternan by alternansucrase (ASR), only reported in *Leuconostoc mesenteroides*), and α-(1→2) glucosidic bonds (only reported in *Ln. mesenteroides*). Depending on the specificity of the glucansucrase enzyme, oligosaccharides can be formed, which are elongated at the non-reducing end, with varying glucosidic linkages.

Besides their precise catalytic mechanism also features determining the linkage specificity of glucansucrases (glucosyltransferases, GTFs) remain to be elucidated. No detailed structural information is yet available for GTF enzymes of glycoside hydrolase family 70 (GH70), but their catalytic domains are closely related to glycoside hydrolases of family 13 (GH13) for which 3D structures and catalytic mechanism have been elucidated. Secondary structure prediction studies of the catalytic domain show that glucansucrases possess a $(\beta/\alpha)_8$ barrel structure like glycosidases (including α-amylase, cyclodextrin glycosyltransferase (CGTase), and amylosucrase of family GH13. Most members of this family hydrolyze / synthesize α-(1→4) and α-(1→6) glucosidic linkages. The $(\beta/\alpha)_8$-barrel structure motif is characterized by the presence of 8 β sheets located in the core of the protein alternated with 8 α-helices located at the surface of the protein. The $(\beta/\alpha)_8$-barrel of glucansucrases is circularly permuted. The four conserved regions (I-IV) identified in the catalytic domain of members of the α-amylase family can also be found in the catalytic domain of glucansucrases. However, as a consequence of the circular permutation, region I occurs C-terminal of region II-IV in glucansucrase enzymes. The seven amino acid residues that are fully conserved in the α-amylase family are also present (except for one residue) in the glucansucrase family (Chapter 1).

The largely similar structural features of members of family GH13 and the glucansucrase family (GH70) provide an excellent starting point for investigations of structure/function relationships in GTF enzymes. Alignments between both families will allow identification of targets for mutagenesis aiming to alter general activity, linkage specificity and hydrolysis/transferase activity ratios in GTF enzymes. This eventually will lead to a better understanding of the reaction mechanism, elucidation of features determining what type of linkages are synthesized, and the hydrolysis/transferase ratios of GTF enzymes.

The aims of the research described in this thesis were (i) to analyze a possibly wider distribution of glucansucrase genes and enzymes in lactobacilli, (ii) to isolate and characterize the glucansucrase genes and enzymes from different lactobacilli and analysis of their oligosaccharides and glucan products (iii) to identify GTF regions important in glucan structure determination (by applying site-directed mutagenesis) (iv) to obtain a better understanding of GTF features determining linkage specificity within the oligo- and polysaccharide products synthesized.

Chapter 1 presents a review, including the results reported in the different chapters of this thesis, of current knowledge of the different LAB glucansucrase genes, enzymes, reactions catalyzed and glucan products synthesized, as well as a comparison between structural features of family GH13 and glucansucrase family 70 (GH70).

The isolation of a novel glucansucrase gene (*gtfA*), the first from a *Lactobacillus* strain is presented in **Chapter 2.** Previous work had shown that *Lactobacillus reuteri* 121 was capable of synthesizing a fructan and a unique glucan polymer containing α-(1→4),

α-(1→6) and α-(1→4,6) glucosidic bonds (van Geel-Schutten *et al.*, 1999). Using degenerate primers based on homologous boxes of glucansucrases from *Leuconostoc* and *Streptococcus* sp., part of a glucansucrase gene was isolated from the chromosomal DNA of *Lb. reuteri* 121. Using molecular techniques (inverse PCR) the complete *gtfA* gene (5343 bp in size!) was isolated. The deduced amino acid sequence of *gtfA* revealed the presence of a relatively large variable N-terminal domain, and a relatively short C-terminal domain. Surprisingly, upstream of *gtfA* another (part of a) *gtf* gene was present designated *gtfB*. Although with difficulties, due to the rather large size of the *gtfA* gene, its cloning and expression in *E. coli* and subsequent purification was achieved successfully. The heterologously produced purified GTFA enzyme synthesized the same glucan polymer, containing large amounts of a α-(1→4) together with α-(1→6) and α-(1→4,6) glucosidic bonds ("reuteran"), as the *Lb. reuteri* 121 strain. Furthermore, the N-terminal amino acid sequence of GTF purified from culture supernatants of *Lb. reuteri* strain 121 was identical to the deduced amino acid sequence of the *gtfA* gene product. These data show that the GTFA enzyme is capable of synthesizing its complete glucan product from sucrose, and is also responsible for introducing various glucosidic linkages and branches in this glucan. This is the first demonstration of the isolation of a glucansucrase gene from a *Lactobacillus*, encoding an enzyme (GTFA, reuteransucrase) capable of synthesizing α-(1→4) glucosidic linkages.

Chapter 3 describes a biochemical and molecular characterization of reuteransucrase (GTFA) of *Lb. reuteri* 121. The GTFA pH optimum of pH 4.7 was comparable to that of other glucansucrases. Striking features of GTFA are its high temperature optimum of 50 °C, its relatively high residual activity at lower temperatures, and its strong sensitivity to EDTA and Ca^{2+} ions, especially in the transferase reaction. Alignments of the GTFA sequence with glucansucrase from *Streptococcus* and *Leuconostoc* were used to identify residues critical for activity. Mutations in the putative catalytic residues, Asp1024Asn, Glu1061Gln and Asp1133Asn, resulted in 300-1000 fold reduced specific activities. The relatively large N-terminal variable region and relatively short glucan binding domain compared to glucansucrases from *Streptococcus* and *Leuconostoc* sp. raised questions about the precise role of the N- and C-terminal domains in GTFA.

The large N-terminal variable domain (702 amino acids) could be removed without changing the glucosidic linkage specificity of GTFA. Quite to our surprise, the transferase activity (initial rate) of the enzyme(s) with N-terminal deleted variable region increased three to four fold, whereas hydrolysis activity decreased. However, after prolonged incubation of the mutant enzyme with sucrose, resulting in complete sucrose conversion, the distribution of hydrolysis and transferase products was similar to wild type. Conceivably, the large N-terminal domain in the wild type protein causes steric hindrance to the growing glucan chain, its deletion resulting in a strongly increased transferase activity. Subsequent deletion of the atypical repeats (YG-repeats) present in the short C-

terminal domain of GTFA (267 amino acids) resulted in strongly decreased affinity for sucrose in the transferase reaction. At the sucrose concentrations used, no saturation was achieved with the mutant enzyme. Furthermore, these C-terminal repeats were shown to be involved in glucan binding.

The activities of GTFA (and deletion mutants) with sucrose alone, and together with the acceptor substrates maltose and isomaltose, were also investigated, showing that after prolonged incubation the distribution of products of the mutant enzymes was similar to wild type. Analysis of oligosaccharides synthesized by GTFA showed that the α-(1→4) and α-(1→6) glucosidic linkages present in the GTFA glucan product were also conserved in its acceptor reaction products. Product specificity, including glucosidic bond specificity and glucan sizes, are thus determined by properties of the GTFA catalytic domain.

In **chapter 4** the isolation and subsequent molecular and biochemical characterization of a second reuteransucrase (GTFBIO), isolated from a probiotic *Lb. reuteri* "BioGaia" strain, is presented. *Lb. reuteri* strain "BioGaia" was isolated as a pure culture from a Reuteri™ Tablet purchased from the BioGaia Company. This tablet contains 100 million active *Lactobacillus reuteri* cells and assists to restore the balance of microflora in the gastrointestinal tract. This *Lb. reuteri* strain was shown to colonize the human stomach, duodenum, and ileum. Furthermore, a clear immunomodulating effect in the human gut was observed (Valeur *et al.*, 2004). Using a similar approach as used for isolation of *gtfA* (degenerate primers and inverse PCR) the complete *gtfbio* gene (5343 bp) was isolated and cloned into *E. coli*. The expression of the *gtfBio* gene in *E. coli* was successful. The pH optimum of GTFBIO was similar (pH 5.0) to that of GTFA. However, the pH optimum was 15 degrees lower (35 °C) when compared to GTFA. The enzyme encoded by *gtfBio* of *Lb. reuteri* BioGaia expressed in *E. coli* synthesized virtually the same glucan polymer, containing large amounts of a α-(1→4) together with α-(1→6) and α-(1→4,6) glucosidic bonds ("reuteran") as *Lb. reuteri* BioGaia. There was a marked difference in the amount of α-(1→4) linkages (~70%) present in the glucan synthesized by GTFBIO when compared to the amount of α-(1→4) linkages (~45%) present in the glucan synthesized by GTFA of *Lb. reuteri* 121. GTFBIO synthesized a glucan with the largest amount of α-(1→4) glucosidic linkages reported to date. GTFBIO was also 50% more efficient in using isomaltose (glucose-α-(1→6)-glucose) as acceptor reaction substrate than GTFA. Another difference between these two reuteransucrases was the hydrolysis/transferase activity ratio. Although both enzymes clearly convert sucrose into large amounts of glucan products upon prolonged incubation, GTFBIO displayed only hydrolytic activity and no transferase activity (initial rates). Upon sucrose depletion, GTFBIO had converted more than 50 % of this substrate into glucose (due to sucrose hydrolysis). In case of GTFA, only 20 % of sucrose had been converted into glucose upon substrate depletion. GTFA and GTFBIO are 69% identical and 80% similar at the amino

acid level, but synthesize different glucan polymers and have different hydrolysis/transferase ratios (initially as well as after substrate depletion). Therefore, GTFA and GTFBIO are interesting candidates to examine structural differences (alignments, hybrid enzymes, site-directed mutants) determining the larger amount of α-(1→4) bonds synthesized by GTFBIO in its products and its higher hydrolytic activity.

The different glucansucrase enzymes of LAB display high sequence similarity. Their linkage specificity is probably caused by relatively small differences in their putative acceptor sugar-binding subsites. Based on a comparison with sugar-binding acceptor subsites in family GH13 enzymes, the locations of two regions putatively involved in acceptor substrate binding in GTF enzymes were identified. A third (putative) acceptor substrate binding region was identified on basis of earlier mutagenesis studies with different GTF enzymes, involving amino acid residue 1138 and 1142 (GTFA, *Lb. reuteri* 121 numbering), determining the solubility of the glucan products and the ratio of [α-(1→3) versus α-(1→6)] glucosidic linkages present (Shimamura *et al.*, 1994, Monchois *et al.*, 2000b, Remaud-Simeon *et al.*, 2000).

Based on alignments of the three putative acceptor sugar-binding subsites, amino acid residues conserved in other glucansucrase, but not conserved in both reuteransucrase enzymes, were identified as targets for site-directed mutagenesis experiments as described in **Chapter 5.** A range of site-directed mutants were constructed in GTFA of *Lb. reuteri* 121 and used to study the effect of amino acid changes on enzyme characteristics. A triple amino acid mutation (N1134S:N1135E:S1136V) in a region immediately next to the catalytic Asp1133 (putative transition state stabilizing residue) converted GTFA from a mainly α-(1→4) (~45%, reuteran) to a mainly α-(1→6) (~80%, dextran) synthesizing enzyme. Affinity for the substrate sucrose decreased 10 fold upon introduction of this triple amino acid mutation. The spectra of products formed from sucrose plus maltose or isomaltose (upon depletion of sucrose) changed drastically. When incubated with sucrose plus maltose, the wild type GTFA and the triple mutant enzyme both converted 60% of maltose into oligosaccharide products. However, the triple mutant synthesized an oligosaccharide with a degree of polymerization (DP) of 4, identified with enzymatic digestion as α-(1→6) -panose, which was not synthesized at all by wild type GTFA. Oligosaccharide yields with sucrose and isomaltose as acceptor reaction substrate increased 2 fold (from 30 to 60%) for the triple amino acid mutation. Furthermore, isomaltotriose and isomaltotetraose oligosaccharides were synthesized, present in minor amounts, as products of wild type. The subsequent introduction of mutation P1026V:I1029V, involving two residues located in a region next to the catalytic Asp1024 (nucleophile), resulted in synthesis of an α-glucan containing only a very small percentage of α-(1→4) glucosidic linkages (~5%) and a further increased percentage of α-(1→6) glucosidic linkages (~85%). The quintuple GTFA mutant showed the same oligosaccharide yields with sucrose and isomaltose as acceptor substrate as the triple

mutant. However, this quintuple mutant synthesized larger amounts of isomaltotetraose. Furthermore, this quintuple mutant synthesized isomaltopentaose and isomaltohexaose.

The mutant GTFA enzymes thus displayed changed glucosidic linkage specificity in the synthesis of glucans (from sucrose) and in oligosaccharide products synthesized from (iso)maltose and sucrose. The variations in glucosidic linkage specificity observed in products of different glucansucrase enzymes indeed appear to be based on relatively small differences in amino acid sequences in their sugar-binding acceptor subsites. Strikingly, the hydrolysis and transferase activities (after substrate depletion) of the mutants mentioned above were affected only to a minor extent.

Another site-directed mutant (H1065S:A1066S) was constructed with the aim to make GTFA more similar to GTFBIO; this largely succeeded and resulted in complete loss of transferase activity (initial rate) in the mutant enzyme. The H1065S:A1066S mutant enzyme showed the same product spectrum as GTFA when incubated with sucrose and the acceptor substrates (iso)maltose (analyzed upon sucrose depletion). The larger amounts of α-(1→4) glucosidic linkages synthesized by GTFBIO compared to GTFA can not be explained by differences in amino acid sequence in the three (putative) acceptor sites. Two sites are identical in both enzymes and mutation of the third site did not affect the nature of the glucosidic linkages in the glucan and oligosaccharide products. Conceivably, more GTF regions are involved in determining the nature and ratio of glucosidic linkages synthesized. Synthesis of hybrid reuteransucrase enzymes may lead to a better understanding of what determines the variation in α-(1→4) specificity in these enzymes. Alternatively, 3D structures of these enzymes may be needed to identify the essential differences.

Chapter 6 describes an efficient method for identification of (partial) glucosyltransferase genes in *Lactobacillus* strains using degenerate primers based on conserved regions in the catalytic domains of glucansucrases from *Leuconostoc*, *Streptococcus* and *Lactobacillus* sp. Fragments of 10 putative glucansucrase genes were identified in eight members of the genus *Lactobacillus* (five different *Lb. reuteri* strains, *Lactobacillus fermentum*, *Lactobacillus sakei* and *Lactobacillus parabuchneri*) were isolated. The different strains also synthesized glucan polymers from sucrose. Glucansucrase genes and enzymes thus appear to occur widespread in members of the genus *Lactobacillus*.

Chapter 7 reports the first time isolation and characterization of six different full-length dextransucrase and mutansucrase genes and enzymes of various *Lactobacillus* spp., and the glucan products synthesized, with mainly α-(1→6) and/or α-(1→3) glucosidic linkages. The recombinant GTFB of *Lb. reuteri* 121 was not active under the conditions tested. Its inactivity may be caused by the aberrant amino acid sequence at the start of its catalytic core. The highly conserved motif "IN**G**QY**Y**" indicating the start of the catalytic

core in GTF enzymes is absent in GTFB. In the supernatant of *Lb. reuteri* 121 grown cultures only the GTFA enzyme was found (under the growth conditions tested).

The active glucosyltransferase enzymes, GTF180 (dextran) and GTFML1 (mutan), characterized from *Lb. reuteri* 180 and *Lb. reuteri* ML1 display high sequence similarity with GTFA of *Lb. reuteri* 121 (reuteran). Interestingly, all *Lb. reuteri* GTFs (including both reuteransucrase enzymes GTFA and GTFBIO) have relatively large N-terminal variable regions and relatively short putative glucan binding domains with conserved and less conserved YG-repeating units. The three other GTF enzymes (GTFKg15, GTFKg3 and GTF33) isolated from *Lb. sakei* Kg15, *Lb. fermentum* Kg3 and *Lb. parabuchneri* 33 contain smaller variable regions and larger putative glucan binding domains when compared to the *Lb. reuteri* GTF enzymes.

Major breakthroughs achieved in this thesis are (i) proficient expression of the relatively large size *Lactobacillus* GTF enzymes in *E. coli* and development of relatively easy and fast GTF enzyme purification protocols. Furthermore, different analytic tools have been developed and implemented: (ii) detailed kinetic analysis, with hydrolysis, transferase and total enzyme activity clearly distinguished; (iii) measurement of products synthesized after substrate depletion (end point conversion) by Dionex analysis; (iv) analysis of oligosaccharide products synthesized by Dionex analysis; (v) identification of purified oligosaccharide products by enzymatic degradation followed by Dionex analysis; (vi) detailed analysis of glucan polymers using methylation, NMR and HPSEC-MALLS studies. The combination of these assay methods has allowed a detailed analysis of wild type and mutant GTF enzymes from lactobacilli.

The results presented in this thesis show that the same variety of *gtf* genes, GTF enzymes and dextran and mutan products are synthesized by members of the genus *Lactobacillus* as previously reported for the genera *Leuconostoc* and *Streptococcus*. Moreover, reuteran synthesis has only been reported for *Lb. reuteri* 121 and *Lb. reuteri* BioGaia, both employing specific reuteransucrase enzymes. The synthesis of alternan and glucan with mainly α-(1$\rightarrow$2) linkages, however, has only been observed in *Leuconostoc* strains.

GTFA amino acid residues important in determining initial hydrolysis/transferase activity ratios have been identified in this study. Furthermore, amino acids residues crucial for glucosidic linkage type specificity of reuteransucrase enzymes in both glucan and oligosaccharide products have been identified.

Future perspectives

Although a detailed analysis has been carried out of the glucosidic linkages present in the glucan products synthesized by the different (mutant) GTF enzymes, the precise glucan structures remains to be elucidated. Also the exact structure of the unique reuteran polymer synthesized by GTFA is currently not known. However, NMR analysis of

enzymatic and chemical degradation products of the reuteran polymer may lead to elucidation of the reuteran structure. A detailed NMR analysis of the different oligosaccharides synthesized by GTFA from iso(maltose) and sucrose is currently in progress and also may serve to identify the reuteran degradation products. The preliminary data confirm the structures of the DP3 and DP4 oligosaccharide products (personal communication, Sander van Leeuwen and Hans Kamerling) as presented in this study, determined by enzymatic degradation and Dionex analysis (chapters 3 and 5).

The experimental data presented in this thesis also confirm the postulated structural similarity between glycoside hydrolase familes 13 and 70 (MacGregor *et al.*, 1996): mutations in similar regions in both enzymes are important in acceptor binding and glucosyl transfer. However, the knowledge of primary sequences of glucansucrases alone is not sufficient for elucidation of the precise mechanism of enzyme catalysis. 3D structural information on enzymes of family GH70 will be vital for a complete understanding of the mechanism of action of these intriguing enzymes and may also serve to expand the range of glucans and glucooligosaccharides that can conveniently be synthesized. Clear understanding of the structural features in glucansucrase enzymes that determine the nature and ratio of glucosidic linkages, degree of branching and polymer size, eventually may allow production of tailor-made glucan and oligosaccharide products for diverse applications.

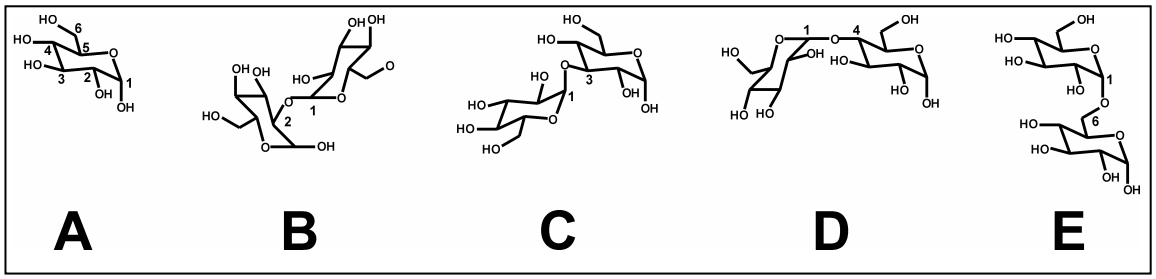
Our expression and purification protocol enabled us to obtain large amounts of GTFA protein for crystallization purposes. Crystals have been obtained of the N-terminally truncated GTFA protein (116 KDa), which so far diffracted only up to 3.5 Angstrom (personal communication, Andreja Vujicic and Bauke Dijkstra). Since the other *Lb. reuteri* GTF proteins can be obtained at the same high degree of purity and are also available as N-terminal truncated enzymes, they are interesting candidates to perform crystallization experiments.

Chapter 9

Nederlandse samenvatting

Melkzuurbacteriën worden van oudsher gebruikt om de houdbaarheid van levensmiddelen te verlengen. Zo worden voor de bereiding van wijn, bier, brood, worst, kaas, yoghurt, augurken en zuurkool melkzuurbacteriën gebruikt. Tijdens het productieproces met melkzuurbacteriën ontstaan naast melkzuur, waaraan de producten hun kenmerkende zure smaak te danken hebben, verscheidene andere stoffen, die de geur, smaak en houdbaarheid van de eindproducten beïnvloeden. Melkzuurbacteriën zijn in staat om een grote verscheidenheid aan exopolysachariden (EPS) te vormen. Deze EPS zijn lange suikerketens (polysachariden) die aan de buitenkant (exo) van bacteriën gemaakt worden. Ze zijn opgebouwd uit aan elkaar gekoppelde suiker eenheden (monosachariden). Homopolysachariden zijn EPSen die opgebouwd zijn uit één type suiker (glucose of fructose) en worden gemaakt door één enzym, een sucrase.

Homopolysachariden kunnen onderverdeeld worden in fructanen en α-glucanen en zijn opgebouwd uit respectievelijk fructose en glucose eenheden. De α-glucanen worden gemaakt vanuit sucrose (tafelsuiker), bestaande uit een glucose en fructose eenheid, door glucansucrase (GTF) enzymen. Deze splitsen sucrose en koppelen de glucose eenheden aan elkaar tot een groot glucanpolymeer, waarbij fructose vrijkomt. De glucose eenheden kunnen aan elkaar gekoppeld worden op verschillende manieren, waardoor ze steeds anders aan elkaar vastzitten (Fig. 1).



Figuur 1. Overzicht van de mogelijke bindingen tussen twee glucose eenheden in α-glucanen. **A** ongebonden glucose, **B** α-(1→2) binding, **C** α-(1→3) binding, **D** α-(1→4) binding en **E** α-(1→6) binding.

De rol van α-glucanen in melkzuurbacteriën is niet helemaal duidelijk. Wel is bekend dat deze polysachariden een rol spelen bij de hechting aan oppervlakten (bijvoorbeeld bij de hechting aan het tandoppervlak en de vorming van tandplak door *Streptococcus* bacteriën).

Glucanen hebben verschillende toepassingen in de levensmiddelen- en farmaceutische industrie (bijvoorbeeld in broodproducten en als volume vergroters in bloedzakken).

Het werk beschreven in dit proefschrift betreft met name glucansucrases van één groep melkzuurbacteriën, de lactobacillen. In onze darmen leven ongeveer 100.000

miljard bacteriën van wel 400 verschillende soorten. Hieronder zitten zowel nuttige als schadelijke bacteriën. Al deze bacteriën samen worden darmflora genoemd. Zodra de slechte of ziekmakende bacteriën de overhand krijgen, kun je last krijgen van gasvorming, diarree, verstopping of infecties. De goede bacteriën kunnen weer de overhand krijgen door extra hoeveelheden van deze bacteriën (zogenaamde probiotica) in te nemen. Lactobacillen zijn goede melkzuurbacteriën en hebben naast de eerder beschreven conserverende rol ook gezondheidsbevorderende eigenschappen en kunnen zo helpen bepaalde ziektes te voorkomen. Een aantal gezondheidsbevorderende eigenschappen die worden toegeschreven aan melkzuurbacteriën zijn bijvoorbeeld (i) het remmen van de groei van ziekte verwekkende bacteriën (ii) het versterken van het immuunsysteem (iii) het verlagen van het cholesterol gehalte en (iv) het voorkomen van bepaalde vormen van kanker.

Bepaalde oligosachariden (korte suikerketens) en polysachariden, zoals glucanen (bestaande uit een grote hoeveelheid suikers) hebben prebiotische eigenschappen. Dat wil zeggen dat deze producten niet door ons lichaam afgebroken worden, maar wel door de in onze darmen aanwezige gezonde bacteriën, bijvoorbeeld bifidobacteriën en lactobacillen. Zo stimuleren prebiotica de groei van deze goede bacteriën. De combinatie van de probiotische *Lactobacillus* stammen en prebiotische glucanen of oligosacharide producten die ze synthetiseren hebben een potentiële toepassing als zogenaamde synbiotica (combinatie van pro- en prebiotica).

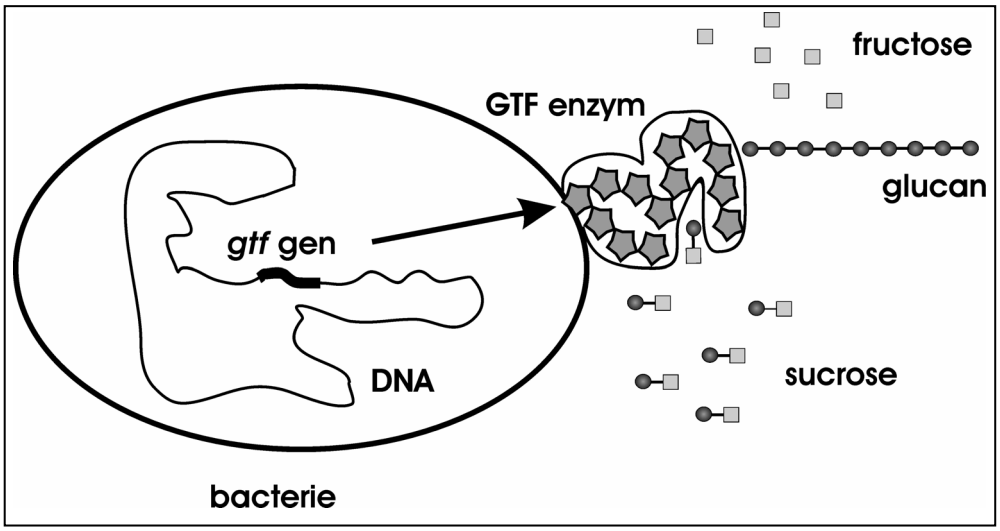
Enzymen zijn eiwitten. Ze zijn opgebouwd uit aminozuren. Er zijn ongeveer twintig verschillende aminozuren waaruit een enzym opgebouwd kan zijn. De volgorde en aard van de aminozuren in een enzym bepalen de eigenschappen van het enzym. In de bacterie cel bevindt zich het DNA dat alle genetische informatie (genen) bevat. Elk gen codeert voor een bepaald eiwit (en dus enzym). Zo wordt ieder enzym gemaakt op basis van deze genetische informatie (Fig. 2).

In de afgelopen jaren zijn er een groot aantal glucansucrase genen en bijbehorende enzymen geïsoleerd en gekarakteriseerd. Deze zijn afkomstig uit twee verschillende groepen melkzuurbacteriën, de streptococcen en de leuconostocs. De glucansucrases zijn in staat om glucanen met verschillende bindingen te synthetiseren. Wanneer ze bestaan uit voornamelijk α-(1$\rightarrow$6) bindingen dan worden ze dextranen genoemd, uit α-(1$\rightarrow$3) bindingen dan worden ze mutanen genoemd en als alternerend α-(1$\rightarrow$3) en α-(1$\rightarrow$6) bindingen aanwezig zijn worden ze alternan genoemd.

De bepalende kenmerken van glucansucrases voor het soort binding dat gevormd wordt in het glucan zijn nog niet gevonden. Vergelijking van de aminozuur volgordes van glucansucrases die verschillende soorten bindingen maken zal mogelijk kunnen leiden tot de identificatie van plekken die bepalend zijn voor de bindingen die gemaakt worden.

De doelen van het onderzoek beschreven in dit proefschrift waren: (i) onderzoeken of glucansucrase genen en enzymen wijdverspreid voorkomen in lactobacillen, (ii) het

isoleren en karakteriseren van glucansucrase genen en enzymen uit verschillende lactobacillen en de analyse van de geproduceerde glucanen, (iii) het identificeren van regio's in GTF enzymen die de bindingstypen in de glucanen en gluco-oligosachariden bepalen. Met behulp van de fundamentele kennis (Rijksuniversiteit Groningen) kunnen in de toekomst specifieke producten met verschillende toepassingsmogelijkheden gemaakt worden (TNO Voeding).



Figuur 2. Schematische weergave van glucan productie door een melkzuurbacterie. In de bacterie ligt het DNA. Op dit DNA ligt een glucansucrase gen (*gtf*) dat vertaald wordt in een glucansucrase enzym (GTF, hier voor het gemak maar opgebouwd uit 13 (dezelfde) aminozuren. Dit enzym splitst sucrose (opgebouwd uit een glucose en fructose) en plakt de glucose eenheden aan elkaar tot een glucan (polymerisatie).

Het inleidende **Hoofdstuk 1** beschrijft de actuele kennis over glucansucrase genen, enzymen, de reacties die ze katalyseren en de producten die ze synthetiseren.

Lactobacillus reuteri 121 is in staat was om een fructan en een uniek glucan polymeer te synthetiseren met α-(1→4), α-(1→6) en α-(1→4,6) bindingen. De isolatie van een nieuw glucansucrase gen (*gtfA*), de eerste uit een *Lactobacillus* stam, wordt beschreven in **hoofdstuk 2.** Dit *gtfA* gen uit de *Lactobacillus* stam werd in een andere bacterie gebracht, de *Escherichia coli.* Hierdoor is deze *E. coli* nu ook in staat om het glucansucrase enzym te maken. Het glucanproduct geproduceerd door de *Lb. reuteri* 121 stam en het *E. coli* GTFA werden met elkaar vergeleken. Hieruit bleek dat GTFA verantwoordelijk is voor de synthese van het unieke glucan met veel α-(1→4) bindingen en ook α-(1→6) en α-(1→4,6) bindingen. Dit glucan wordt voor het gemak reuteran genoemd. Het glucansucrase dat dit reuteran maakt, wordt overeenkomstig reuteransucrase (GTFA) genoemd.

In **Hoofdstuk 3** worden de eigenschappen beschreven onder welke omstandigheden het GTFA enzym van *Lb. reuteri* 121 het beste werkt. Verder werden er drie aminozuren van het enzym geïdentificeerd die cruciaal zijn voor de activiteit van GTFA. Verder werd aangetoond dat wanneer grote stukken van het enzym verwijderd werden het nog steeds in staat was om glucanen te maken.

Hoofdstuk 4 beschrijft de isolatie en de karakterisatie van een tweede reuteransucrase (GTFBIO), geïsoleerd uit een probiotische *Lb. reuteri* stam "BioGaia". Deze *Lb. reuteri* stam was geïsoleerd uit een Reuteri™ Tablet zoals het verkocht wordt door het bedrijf BioGaia. Deze tablet bevat 100 miljoen actieve *Lactobacillus reuteri* cellen en helpt het evenwicht van de microflora in de darmen te herstellen. GTFBIO, gecodeerd door het *gtfBio* gen, produceerde wanneer het tot expressie gebracht werd in *E. coli* hetzelfde polymeer, met veel α-(1→4) bindingen en kleinere hoeveelheden α-(1→6) en α-(1→4,6) bindingen, als *Lb. reuteri* BioGaia. Er was wel een duidelijk verschil tussen de hoeveelheid α-(1→4) bindingen in het reuteran gesynthetiseerd door het GTFBIO van *Lb. reuteri* BioGaia (70%) en het GTFA *Lb. reuteri* 121 (45%). GTFBIO en GTFA hebben 69% identieke en 80% vergelijkbare aminozuren, maar maken een verschillend glucan (reuteran) polymeer, daarom zijn deze beide enzymen interessante kandidaten om het verschil in α-(1→4) bindingen te bestuderen.

De verschillende glucansucrase enzymen van melkzuurbacteriën lijken qua aminozuur compositie erg op elkaar. Het verschil in het type binding dat ze maken wordt daarom waarschijnlijk veroorzaakt door kleine verschillen. Door vergelijking van glucansucrases die verschillende bindingen maken, werden aminozuren ontdekt die op een vaste plek aanwezig waren in alle andere glucansucrases, maar niet in beide reuteransucrases. Deze aminozuren werden gericht veranderd in GTFA van *Lb. reuteri* 121. De veranderde GTF enzymen werden gebruikt om de effecten van de betreffende aminozuur veranderingen op de enzym eigenschappen te bestuderen, zoals beschreven in **hoofdstuk 5.** Wanneer drie aminozuren werden gewijzigd, veranderde GTFA van een voornamelijk α-(1→4) (~45%, reuteran) naar een voornamelijk α-(1→6) (~80%, dextran) synthetiserend enzym. Het introduceren van twee extra aminozuur veranderingen, resulteerde in de synthese van een glucan met een heel laag percentage α-(1→4) bindingen (~5%) en een verdere verhoging van het aantal α-(1→6) bindingen (~85%). De veranderde GTFA enzymen laten dus een duidelijke verschuiving zien in de bindings specificiteit van de glucan producten gemaakt uit sucrose. Deze variaties in bindings specificiteit worden dus inderdaad veroorzaakt door kleine verschillen in het enzym.

Hoofdstuk 6 beschrijft een efficiënte methode om glucansucrase genen uit *Lactobacillus* stammen te isoleren. Fragmenten van 10 glucansucrase genen werden gevonden in acht verschillende lactobacillen (vijf verschillende *Lb. reuteri* stammen, een *Lactobacillus fermentum*, een *Lactobacillus sakei* en een *Lactobacillus parabuchneri*). De

verschillende stammen produceerden ook glucan polymeren vanuit sucrose. Glucansucrase genen en enzymen lijken dus wijdverspreid te zijn onder lactobacillen.

Hoofdstuk 7 beschrijft de complete isolatie van zes dextransucrase en mutansucrase genen/enzymen uit de verschillende *Lactobacillus* stammen, beschreven in hoofdstuk 6, en de analyse van de geproduceerde glucanen, met voornamelijk α-(1→6) en/of α-(1→3) bindingen.

De resultaten in dit proefschrift laten zien dat dezelfde variëteit aan *gtf* genen, GTF enzymen en dextran en mutan producten worden gesynthetiseerd door de *Lactobacillus* bacteriën als hiervoor beschreven voor de *Leuconostoc* en *Streptococcus* bacteriën. Bovendien is reuteran synthese alleen beschreven voor *Lb. reuteri* 121 en *Lb. reuteri* BioGaia. De synthese van alternan en een glucan met veel α-(1→2) bindingen is tot nu toe alleen gevonden in *Leuconostoc* stammen.

Verder zijn aminozuren geïdentificeerd die belangrijk zijn voor de activiteit en het bepalen van het bindingstype van zowel het glucan polymeer als de oligosachariden die gesynthetiseerd worden door het GTFA van *Lb. reuteri* 121. Hierdoor is een stap vooruit gemaakt in het ontwerpen en maken van specifieke glucanen vo or doelgerichte toepassingen.

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